THE
BIOLOGICAL BULLETIN

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THE
BIOLOGICAL BULLETIN

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THE ELECTRIC STIMULATION OF THE CHROMATOPHORAL NERVE-FIBERS IN THE DOGFISH

G. H. PARKER

(*From the Woods Hole Oceanographic Institution*[1] *and the Marine Biological Laboratory, Woods Hole, Mass.*)

In the chromatophoral color changes of the common dogfish, *Mustelus canis,* whereby this fish may assume a dark or a light coloration, it has been shown that the dark phase is excited by secretions from the pituitary gland (Lundstrom and Bard, 1932) and that the light one is due to the action of concentrating nerve-fibers (Parker and Porter, 1934). Parker and Porter stimulated these fibers by cutting them transversely, a procedure that was particularly successful when it was carried out near the base of a fin. Under such circumstances a light band quickly appeared on the fin in what was obviously the area of peripheral distribution for the severed nerve-fibers. That the operation had caused no serious disturbance in the blood supply to the region concerned could be shown by a microscopic examination of the living skin in the band itself. By this means an unimpeded flow of blood in the light area could easily be demonstrated. Parker and Porter employed no other method for stimulating the chromatophoral nerve-fibers than cutting them. In the present paper it is intended to show that an electric current from an inductorium is as efficient a means of stimulating these fibers as is their severance.

It was found convenient to experiment on the anterior dorsal fin of the dogfish. This fish, with its dorsal fin uppermost, can be tied to a wooden carrier and kept indefinitely quiescent by providing it with a current of sea water over its gills. Near the root of its dorsal fin and not far from the anterior edge of this structure a cut 5 mm. long, transverse to the fin rays and completely through the fin was made as a means of exciting a peripheral light band. Posterior to this cut and in line with it two moderately large needle holes were made through the fin and about 5 mm. apart. Into each hole was inserted the platinum elec-

[1] Contribution No. 60.

trode from an electric inductorium which, however, was not yet in operation. The whole preparation was allowed to stand for half an hour. During this time a well-developed light band formed in the region of the fin peripheral to the cut but no change was noticeable in that part of the fin that was distal to the two holes. Having been assured that the making of the holes and the insertion into them of the two electrodes had not changed the coloration of the fin, I now started the inductorium. About ten minutes after this had been done a light area began to appear in the fin peripheral to the holes and in 25 minutes it was well pronounced and essentially indistinguishable from that which had resulted from the cutting of the fin (Fig. 1). This test was carried out on four fishes and with similar results in all cases.

When a fin into which electrodes have been inserted is simultaneously stimulated both by making the current and by cutting the nerve-fibers in positions such as have already been described, the formation of the two light bands can be watched step by step. Under such circumstances they will be seen to form at essentially the same rate and to the same degree. Thus there seems to be no real difference in the light bands formed by these two methods and I therefore conclude that stimulation by an electric current is as effective for the concentrating melanophore nerve-fibers as is cutting.

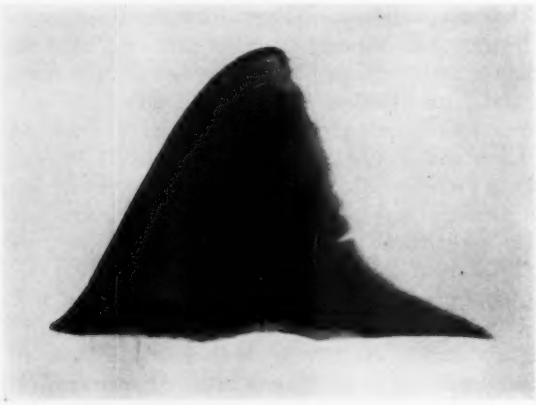

FIG. 1. Photographic side view of the anterior dorsal fin of a dark common dogfish, *Mustelus canis*, which has been stimulated to the formation of light bands by a transverse cut near the anterior edge of the fin and by electric stimulation posterior to this cut. The two holes into which the electrodes were inserted can be seen as dots in line with the transverse cut but posterior to it. The resultant light areas are seen in the fin peripheral to the two regions of stimulation.

In *Fundulus* the cutting of the chromatophoral nerve-fibers stimulates the dispersion of melanophore pigment, not its concentration. Electrical stimulation of these fibers, on the other hand, stimulates concentration, not dispersion (Mills, 1932). In my opinion these conditions are not the result of an exclusive action of the two means of stimulation on different classes of fibers. Both concentrating fibers and dispersing fibers are in all probability excited by each agent. The difference is due, I believe, to the greater ease with which concentrating fibers can be excited electrically and dispersing fibers by cutting. In the dogfish, where only one set of fibers is present, those concerned with pigment concentration, both types of stimulation are effective in bringing about the same reaction. These reactions are best seen in dogfishes that have not reached the stage of maximum darkness but which are only moderately dark. This is probably due to the great effectiveness of the pituitary secretion by which the dogfish melanophores are excited to disperse their pigment as contrasted with the weaker effect of the concentrating nerve-fibers which induce the opposite change (Parker and Porter, 1934).

Conclusion

The concentrating melanophore nerve-fibers of the dogfish can be excited to action whereby the fish may be locally blanched as well through *electric* stimulation as through *mechanical* stimulation (cutting).

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A NUPTIAL SECONDARY SEX-CHARACTER IN FUNDULUS HETEROCLITUS

GEORGE HOWARD PARKER AND HELEN PORTER BROWER

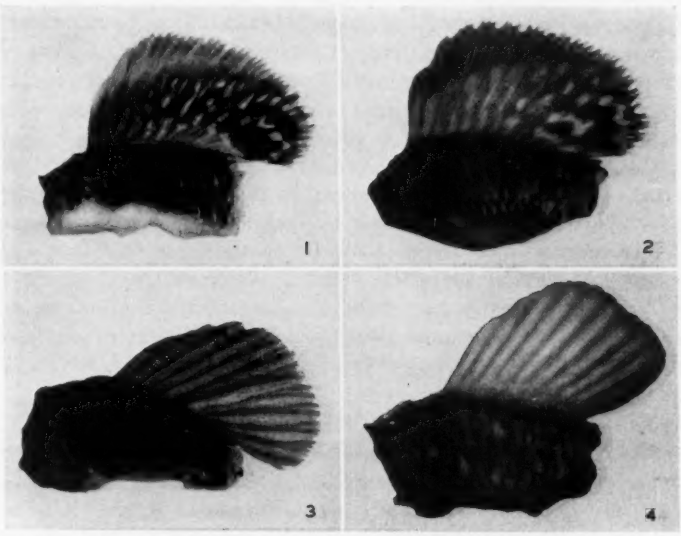
(*From the Biological Laboratories, Harvard University*)

Everyone who has selected *Fundulus* for eggs or sperm is familiar with the striking coloration pattern in the males and especially with the dark splotch in the posterior part of the dorsal fin in this sex (Newman, 1907). This splotch is made up of melanophores and is not without interest. To test its constancy in relation to sex one hundred killifishes were opened during the breeding season (June, 1934) and their sex determined by an inspection of their gonads. Forty-seven of these fishes proved to be males and fifty-three females. All the males exhibited the fin-mark already alluded to and none of the females showed it. Apparently it is a character accurately associated with sex.

It is in all essential respects undisturbed by the change of *Fundulus* from the dark to the light condition or the reverse. Figure 1 shows the dorsal fin of a male fish in the dark phase, and notwithstanding the generally deep tone of the fin, the nuptial mark stands out conspicuously. The anterior three-fourths of the free edge of the fin contains so few melanophores as to appear quite light. The rest of the fin carries the nuptial mark composed of a great abundance of large melanophores. Here and there in this part of the fin are elongated light-spots parallel with the rays. The last few interrays near the posterior edge of the fin are, on the whole, densely black with several striking light-spots that add intensity to the mark. This darkened area and especially its posterior portion is what we have called the nuptial mark. In the light condition of the males the mark, especially its posterior part, is still conspicuous (Fig. 2). In the female the dorsal fin is uniformly dotted over with melanophores which neither in the dark state (Fig. 3) nor in the light one (Fig. 4) show any special concentration.

About a quarter of an hour after a male *Fundulus* has been put into a white-walled illuminated vessel it will have become extremely light-tinted in that the pigment in its skin melanophores will have assumed a high degree of concentration. The nuptial mark, however, will remain under these circumstances conspicuously dark as though the pigment of its melanophores had failed to concentrate. If, however, this spot is examined under the microscope it will be seen to be made up of many melanophores, both large and small, in all of which the pigment is

strongly concentrated. Their number, however, is so great as compared with that in equal areas of other parts of the body that notwithstanding the state of their pigment they form a dark area. But the nuptial spot is not only a rich aggregation of melanophores; it is a region in which a large amount of free melanin occurs. This melanin is in the form of clumps in the tissue spaces of the skin and probably results from the disintegration of color-cells in which it was once contained. In this way the nuptial mark in a light fish remains a con-



Four enlarged views of the dorsal fins of *Fundulus heteroclitus*, two from males and two from females, one in each sex for the light condition and the other for the dark. In each instance the anterior edge of the fin is to the left. Photographs by Dr. F. M. Carpenter.

FIG. 1. Dorsal fin of a male in dark condition.
FIG. 2. Dorsal fin of a male in light condition.
FIG. 3. Dorsal fin of a female in dark condition.
FIG. 4. Dorsal fin of a female in light condition.

spicuous feature even though the pigment in its melanophores may have gone into a concentrated state.

Fundulus, some ten minutes after it has received a subcutaneous injection of adrenalin, will become extremely light in that the pigment in both its large and its small melanophores will have become maximally concentrated. But even in this extreme state the male nuptial mark is still conspicuous and for the same reason as that already given; its melanophores are so numerous that even though their pigment is con-

centrated by adrenalin, they nevertheless constitute a dark area in the fin of a light fish. Their response to adrenalin allies them with the melanophores of the rest of the fish, but puts them in contrast with the yellow and red cells in the so-called "Hochzeitskleid" of *Phoxinus*, which, according to Abolin (1925*a*) and to Giersberg (1930) are not influenced by adrenalin.

The male nuptial mark has an interesting history throughout the year. This history has been followed for some eighteen months on material in part from Woods Hole and in part from the neighborhood of Boston, Massachusetts. No nuptial marks have been noticed in male *Fundulus* from these localities between the months of November and of March. In April the mark begins to appear. It is present in May, is well pronounced in June and July, diminishes in August, and disappears in November. It is therefore quite clearly a mark of the breeding season, as in fact the generally high coloration of the male also is. It does not, however, belong to that group of color changes such as have been studied recently by Wunder (1931) in *Rhodeus* and which momentarily flash up in the male during the breeding act. It is a permanent sign of the whole breeding season and its limitation to one sex suggests that it may result from some male neurohumor produced during that period and acting on a specially receptive area in the dorsal fin of the fish. (See Abolin, 1925*b*.)

Summary

The male of *Fundulus heteroclitus* possesses a secondary sex-character in the form of a melanophoric mark on its dorsal fin to be seen from April to November but not at other times of the year. It may therefore be described as nuptial. It does not occur in the female. It is composed of an unusually dense aggregation of melanophores whose melanin even when concentrated (light phase) forms a conspicuous dark mark.

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RESPONSES OF DEEP-SEATED MELANOPHORES IN FISHES AND AMPHIBIANS

MARY SEARS

(From the Zoölogical Laboratories, Radcliffe College)

Introduction

Although there have been numerous investigations of the activities of the skin melanophores (Parker, 1930), only a few have included the deep-seated melanophores. These (Lieben, 1906; Ogneff, 1908; Hooker, 1912; Allen, 1917; Smith, 1916; Fischel, 1920; Uyeno, 1922; Gilson, 1926; Yamamoto, 1931) suggest little correlation in the activities of the two sets of melanophores. In amphibians, the absence of any marked response in the deep-seated cells to such drugs as adrenalin may have been due to a degenerate condition, since most of the earlier work was carried out after killing the animal. In fish, various reactions have been found, some identical to those in the skin, others the reverse. Because of these results, it seemed well to examine the activities of the deep-seated melanophores in animals in which the behavior of the dermal melanophores had been studied, by the use of the same agents which affect these cells in the skin, to learn whether the reactions of the deep-seated melanophores are comparable with those in the skin. The animals chosen were the common leopard frog, *Rana pipiens* Schreber, the tadpole of *Rana clamitans* Latreille, and the killifish, *Fundulus heteroclitus* L.

This work was carried out at the suggestion of Professor G. H. Parker to whom I am indebted for his constant supervision and advice.

Methods

The three species used, on a black background assume a dark coloration by expansion of their dermal melanophores, and on a white one, a light shade by contraction of these cells. Such conditions may also be induced by hormones, adrenalin producing a light appearance and pituitrin, at least in amphibians, a dark one. In extending these tests to the deep-seated melanophores, white porcelain dishes served for one extreme of background and battery jars painted externally with dull black, the other. In adrenalin tests, the animals were confined in the black jars, and in the pituitrin tests, they were placed in the white dishes, some time before injecting and kept there until they were killed. Throughout

all experiments the animals were illuminated from a western window or by an electric lamp.

As the condition of the deep-seated melanophores may be inspected only upon killing the individual, a group of animals was subjected to the various tests and the internal melanophores of a number of these were examined at intervals during such trials to trace the activities of these cells step by step. Caution was taken that no rearrangement in the

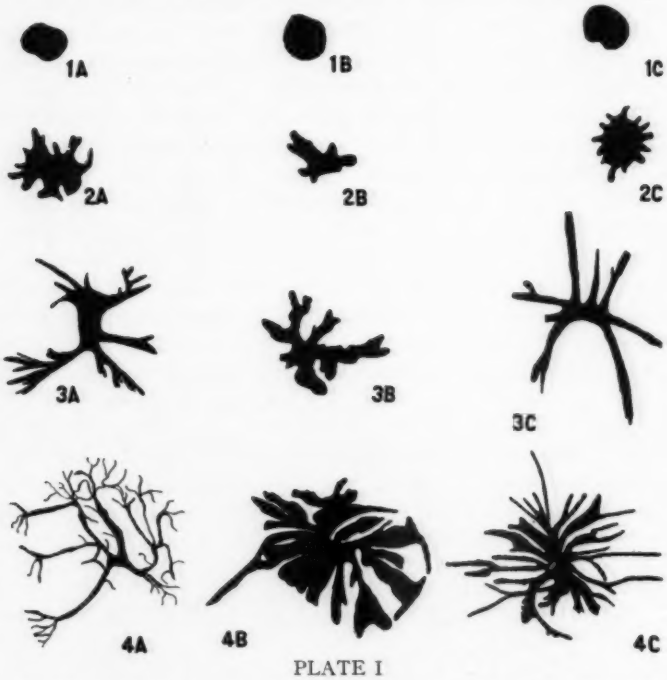


PLATE I

Semidiagrammatic drawings of dermal melanophores from the frog (*A*), the tadpole (*B*), and the killifish (*C*) showing four stages of expansion: 1, punctate condition; 2, stellate condition; 3, incomplete expansion; 4, complete expansion.

position of the pigment within the internal cells occurred during dissection. In frogs and tadpoles, whose dermal melanophores are chiefly under humoral control, no perceptible change appeared after considerable handling or for some hours after pithing. Hence, the state of the internal melanophores within a minute or two of pithing seemed indicative of the living condition. However, in killifish, whose melanophores are almost entirely under nervous control, these cells expanded at once

on manipulation and especially on sectioning their nervous connections. To overcome this, the deep-seated tissues were examined without first destroying the brain, by cutting away the body wall on one side without

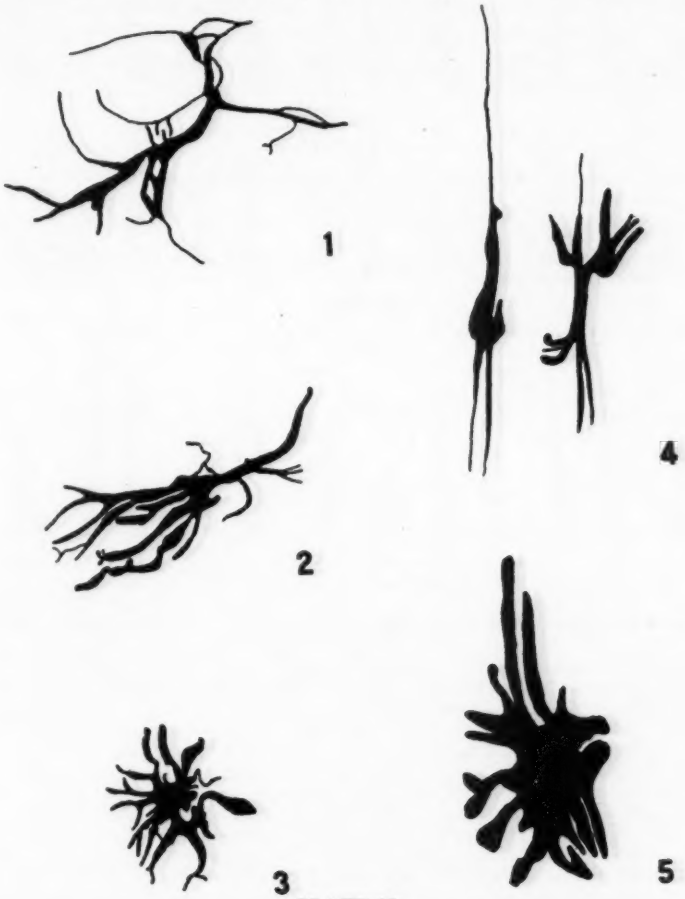


PLATE II

Semidiagrammatic drawings of completely expanded melanophores of the frog in (1) the pleura, (2) the mesenteries, (3) the fascia of the leg muscles, (4) the fascia of the body-wall muscles, and (5) the fascia of the back muscles.

severing the nerves to the parietal peritoneum of the opposite wall or to the visceral peritoneum and mesenteries.

To facilitate a comparison of conditions among the deep-seated me-

lanophores, four conventional stages have been defined as follows: (1) the *punctate* stage, an entirely contracted state in which the cell appears as a minute black dot; (2) the *stellate* stage, or one in which the pigment occupies only the main roots of the cell processes; (3) the stage of *incomplete expansion,* in which the melanin partly fills these; and (4) the stage of *complete expansion,* in which the pigment has spread into the most remote branches (Pl. I).

TABLE I

The variation in the degree of contraction among the melanophores of frogs confined in darkness or on a white illuminated background.

Location of melanophores	Case a74— Kept in dark 20 hours	Case a107— White illuminated surroundings 24 hours	Case a190— White illuminated surroundings 2 weeks
Skin on back	Stellate	Stellate	Stellate
Web	Stellate	Stellate	Stellate
Back muscles	Punctate to stellate	Punctate to incomplete expansion. Mostly stellate.	Punctate
Pleura	Stellate	Punctate	Punctate to incomplete expansion
Pericardium	Punctate to incomplete expansion	Stellate to incomplete expansion	Incomplete expansion
Muscles of body wall	Punctate to stellate	Punctate	Punctate to stellate
Mesenteries	Chiefly punctate to incomplete expansion	Punctate	Punctate

OBSERVATIONS

Frogs

In frogs, the deep-seated melanophores are particularly abundant in the connective tissues of the following regions: the fascia of the leg muscles, of the muscles of the back, of the muscles of the body wall—especially the m. obliquus internus—, the mesenteries, the pericardium, the pleura, and the lining of the sub-dermal lymph sacs. These cells are very similar in general appearance to the melanophores of the skin, with the exception of those in the fascia of the back muscles, which resemble those in tadpole connective tissues (Pl. II).

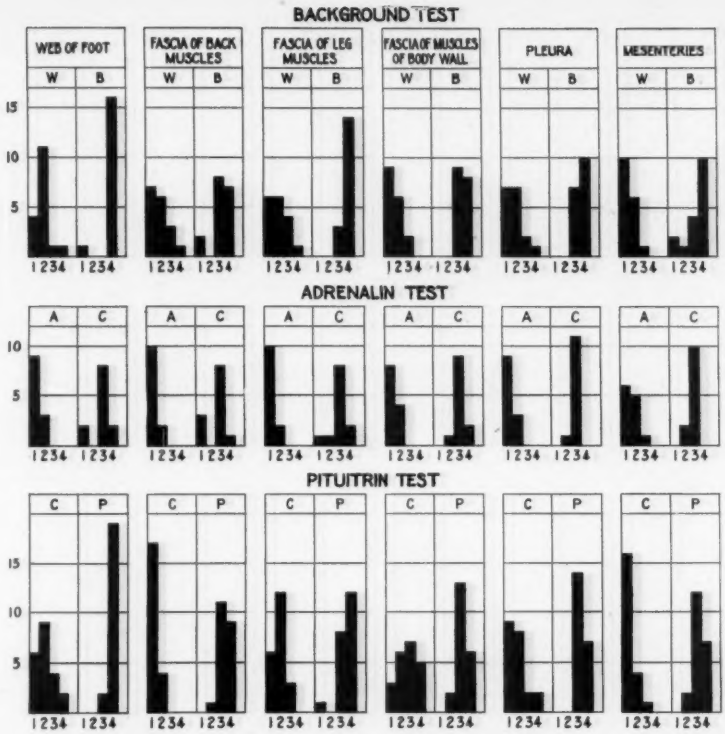


DIAGRAM 1. Tabulation of the effects of background, adrenalin, and pituitrin on the melanophores in six different parts of the frog: the web of the foot, the fascia of the back-muscles, of the leg muscles, and of the muscles of the body-wall, the pleura, and the mesenteries (as shown at the top of the diagram). In the background tests, the shade of the surroundings, white or black, is indicated by *W* or *B* at the head of each column in the upper row. In these tests, as in all the subsequent ones, the results of the factor effecting the more complete contraction are placed to the left and of that producing the less complete to the right in each contrasting pair.

Seventeen frogs were kept on each background for forty-eight hours before observation. In every frog, the amount of melanophore contraction was noted as belonging to one of four stages of contraction of which 1 is maximum contraction and 4 is minimum contraction. The number of cases falling in each class is recorded in black columns over the appropriate figure. Thus, in the web of the foot of frogs on a white background, four animals showed complete contraction, eleven part contraction, one part expansion, and one full expansion: on a black background, one exhibited full contraction and sixteen complete expansion. The remaining tests are all tabulated in a similar manner.

In the adrenalin test, the frogs treated with a 0.5 cc. injection of the drug three hours before examining and their controls with 0.5 cc. tap water injections are designated *A* or *C* respectively at the top of each column in the second row. The twelve frogs in each series were kept on an illuminatd black background for two days before observation.

In the pituitrin tests, the frogs subjected to a 0.5 cc. pituitrin injection three hours before examination and their controls with 0.5 cc. of tap water are distinguished in the diagram by *P* or *C* at the top of each column in the bottom row. The twenty-one frogs in each series were confined in complete darkness for several days before their inspection.

The deep-seated melanophores of frogs confined on a black background for periods extending from a day to a week were predominantly expanded. Yet there were always some cells among them in stages of contraction. This situation was unlike that in the skin where the melanophores were always in a uniform state of expansion.

TABLE II

The rate of response in melanophores to adrenalin. Frogs kept in blackened battery jars under illumination before the injection and until killed.

Location of melanophores	Case a11 1 hr. after 0.5 cc. adrenalin	Case a53 2 hrs. after 0.5 cc. adrenalin	Case a69 3 hrs. after 0.5 cc. adrenalin	Case a112 27 hrs. after 0.5 cc. adrenalin
Skin on back	Punctate	Punctate	Punctate	Stellate
Web	Punctate	Punctate to few stellate	Punctate	Stellate
Leg muscles	Complete expansion	Incomplete expansion. Few punctate.	Punctate	Punctate to incomplete expansion
Pleura	Complete expansion	Incomplete expansion	Stellate	Stellate to incomplete expansion
Pericardium	Complete expansion	Complete expansion	Incomplete to complete expansion	Stellate to complete expansion with proximal concentration
Muscles of body wall	Complete expansion	Incomplete expansion. Some punctate.	Punctate	Punctate
Mesenteries	Complete expansion	Incomplete expansion to few punctate	Punctate	Stellate
Back muscles	Complete expansion	50% punctate, 50% incomplete to complete expansion	Punctate. Occasional stellate.	Chiefly punctate. Some incomplete expansion.

In three individuals placed in a white dish for a day, there was a marked trend toward the contracted state among the internal melanophores. (Table I, third column.) As in the frogs kept on a dark background, there were all stages in the proximal withdrawal of the pigment. Likewise, animals kept on a white background for a week or

more showed great variety in the amount of contraction. (Table I, fourth column.) However, a greater general contraction usually existed with the longer period in white surroundings, suggesting a more sluggish response in the internal melanophores. Though not extreme, the contrast in the position of the pigment of these cells was evident in seventeen frogs kept in white dishes for at least two days, and in a like number confined in black jars for a similar period. (Diagram 1, top row.)

TABLE III

The response of melanophores to pituitrin. See also Table II, fifth column, for condition such as probably existed before the pituitrin injection in a113. Also, see Table I, second and third columns, for the condition of melanophores such as probably existed before the injections of pituitrin in a99 and a105 respectively.

Location of melanophores	Case a113—Pituitrin following adrenalin	Case a99—Pituitrin after 24 hrs. in dark	Case a105—Pituitrin after 2 days on white illuminated background
Skin on back	Complete expansion	Complete expansion	Complete expansion
Web	Complete expansion	Complete expansion	Complete expansion
Muscles of leg	Incomplete expansion	Incomplete expansion	Incomplete expansion
Muscles of back	Incomplete expansion	Incomplete expansion	Incomplete expansion
Pericardium	Incomplete to complete expansion	Complete expansion	Incomplete expansion
Pleura	Incomplete expansion	Incomplete expansion	Stellate to incomplete expansion
Mesenteries	Punctate to incomplete expansion	Incomplete to complete expansion	Incomplete expansion
Muscles of body wall	Incomplete expansion	Incomplete to complete expansion	Incomplete expansion

Adrenalin, which acts promptly and invariably on the dermal melanophores, also effected a slower contraction of the internal pigment cells. An injection of 0.5 cc. of a 1:1,000 solution of adrenalin chloride (Parke, Davis and Company) in the dorsal lymph spaces of eight frogs kept on a dark background for some days to insure expansion of these cells, evoked no reaction in the deep-seated melanophores for more than an hour (Table II, second column). Yet, the same dosage always caused the skin to become extremely light-colored within twenty

minutes. While a considerable percentage of the melanophores in the fascia of the back muscles of seven frogs contracted within an hour after an injection of 0.8 to 1.0 cc. of adrenalin, the effect of increasing the dose on the deep-seated melanophores was not pronounced. If, however, two hours elapsed between a 0.5 cc. injection and the inspection of the internal tissues (Table II, third column), as noted in four cases, at times as many as half the melanophores were entirely contracted, with a greater number among the pigment cells in the fascia of the back muscles. On increasing the period between the injection and the subsequent examination to three hours (Table II, fourth column), the contracted phase became general save in the pericardium. Here, the pigment migrated proximally only after many hours, as was noted in three or four frogs kept alive for nearly a day after the injection (Table II, fifth column). The dose of adrenalin was controlled in every case by an equal one of tap water. Frogs so treated never exhibited as great a contraction of the melanophores as those with adrenalin (Diagram 1, middle row). In fact, the stage of expansion was essentially similar to that in frogs kept in black jars without injections.

As an expansion of the melanophores is usual in the laboratory, and as their contraction is brought about so slowly, it was a bit uncertain in the pituitrin tests, whether a contraction of these cells had occurred in the individual frogs before injecting the drug effecting expansion. Since adrenalin was certain to produce a general proximal migration of pigment, it at first seemed desirable to inject it the day before the pituitrin. This procedure produced a complete expansion of the internal melanophores in eleven frogs, three hours after the injection of 0.5 cc. pituitrin (Parke, Davis and Company—"Obstetrical"). As an interaction of the drugs might have caused this, another test became preferable. Darkness was quicker and more certain in obtaining a contraction of the internal melanophores than white illuminated surroundings (Table I, second and third columns). In frogs confined in the dark for several days, a 0.5 cc. injection of pituitrin effected a more or less complete expansion in about three hours (21 cases) (Table III). In controls with the same amount of tap water, the melanophores were predominantly in the punctate or stellate condition (Diagram 1, bottom row).

Tadpoles

In the tadpoles of *Rana clamitans* in their second year, stubby relatively unbranched melanophores are numerous in most connective tissues, particularly in the peritoneum, the mesenteries, the pleura, and the pericardium (Pl. III).

When the tadpoles were confined in black jars for a day or more, the sub-epidermal melanophores almost always (22 out of 25 cases) exhibited a uniform state of maximum expansion. This condition was extended to the deep-seated melanophores in the pleura, the pericardium, the mesenteries, and, to a certain extent, to those of the peritoneum. In the latter tissue, however, in about half the tadpoles, some of these cells were in the stellate condition, while in one case the greater proportion of these was punctate.

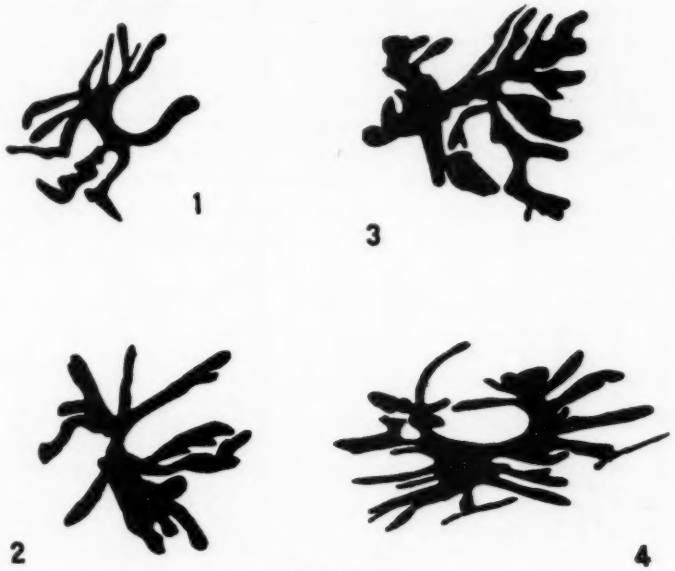


PLATE III

Semidiagrammatic drawings of completely expanded melanophores of tadpoles in (1) the pleura, (2) the mesenteries, (3) the peritoneum, and (4) the pericardium.

If tadpoles were kept in white dishes for a similar period, the sub-epidermal melanophores were usually (23 out of 25 cases) entirely contracted. Among the internal melanophores the amount of contraction which took place under these circumstances was not so great. Except for the melanophores of the pleura, which were predominantly contracted, there were many which exhibited a more or less distal distribution of pigment (Diagram 2, top row).

In larvæ, whose deep-seated melanophores responded as promptly as those in the skin to changes in background, adrenalin was a slower agent for contracting such cells than in the adult frog. Three hours after a

0.3 cc. injection in a 1:1,000 solution into the body cavity, the subepidermal melanophores ranged from the punctate stage to that of incomplete expansion and the internal cells remained for the most part completely expanded. Not until five hours (26 cases) after such a dosage were the majority of the skin melanophores contracted and even then a large percentage were in the stellate condition. Essentially the same situation was found in the internal melanophores. The proportion of animals with these cells in a punctate or stellate condition was greater than in tadpoles on a white background. With longer periods between the injection and the inspection of the animal, no great change occurred in the amount of contraction, and with a larger dose, the animal died within twenty-four hours. In controls with tap water, all the melanophores save a few in the peritoneum of about one-third the animals were completely expanded (Diagram 2, middle row).

With 0.3 cc. of pituitrin injected five hours before examination, the melanophores of tadpoles kept in white dishes were almost always (20 out of 25 cases) in the completely expanded phase. In the controls with tap water, there were only a few (5 out of 25 cases) with completely expanded melanophores (Diagram 2, bottom row).

Fundulus

When compared with the number of deep-seated melanophores in

DIAGRAM 2. Tabulation of the effects of background, adrenalin, and pituitrin on the melanophores in five different parts of the tadpole: the dermis, the pleura, the pericardium, the mesenteries, and the fascia of the body wall muscles (as shown at the top of the diagram).

In the background tests, the shade of the surroundings, white or black, is indicated by *W* or *B* at the head of each column in the upper row. In these tests, as in all subsequent ones, the results of the factor effecting the more complete contraction are placed to the left and that producing the less complete to the right in each contrasting pair. Twenty-five tadpoles were kept on each background for forty-eight hours before observation. In every tadpole, the amount of melanophore contraction was noted as belonging to one of four stages of contraction of which 1 is maximum contraction and 4 minimum contraction. The number of cases falling in each class is recorded in black columns over the appropriate figure. Thus, in the dermis of tadpoles on a white background, 23 showed complete contraction, 1 part contraction, and 1 part expansion: on a black background, 22 exhibited complete expansion, 2 part expansion and 1 part contraction. The remaining tests are all tabulated in a similar fashion. In the adrenalin tests, both the tadpoles treated with a 0.3 cc. injection of the drug five hours before examining and their controls with 0.3 cc. tap water injections are designated *A* or *C* respectively at the top of each column in the second row. The 26 tadpoles in each series were kept on an illuminated black background for two days before observation. In the pituitrin tests, the tadpoles subjected to a 0.3 cc. pituitrin injection five hours before examination and their controls with 0.3 cc. of tap water are distinguished in the diagram by *P* or *C* at the top of each column in the bottom row. The 26 tadpoles in each series were confined in white dishes for several days before their final inspection

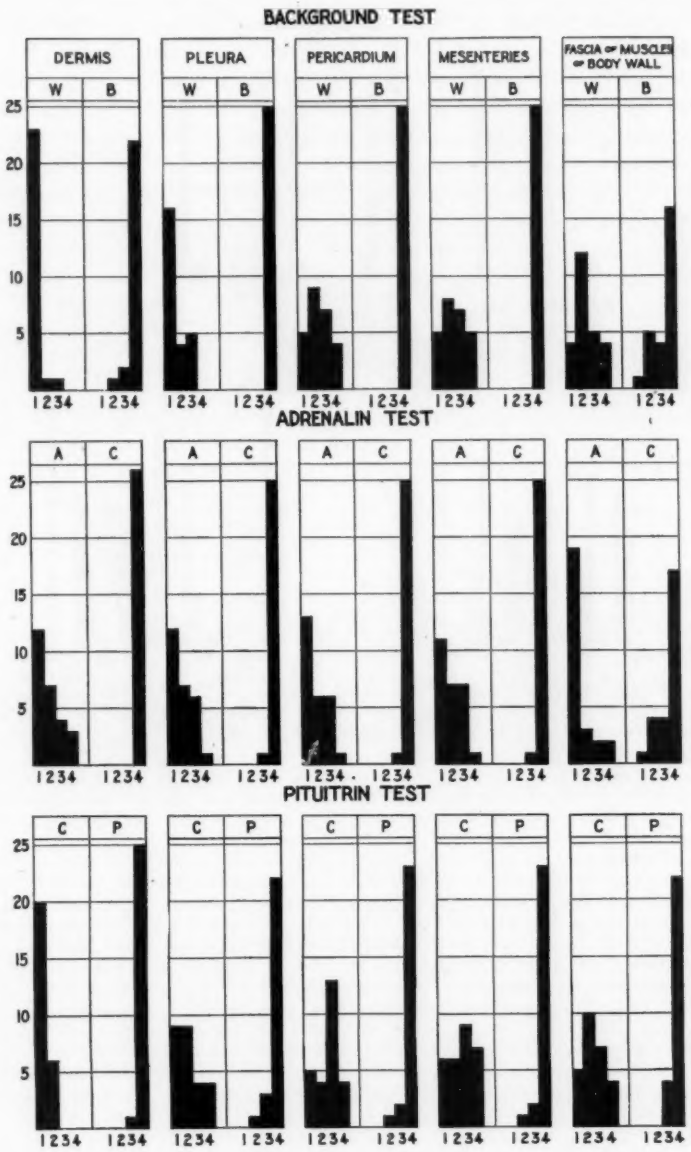

BACKGROUND TEST
DERMIS
PLEURA
PERICARDIUM
MESENTERIES
FASCIA or MUSCLES or BODY WALL
W
B
25
20
15
10
5
1234
ADRENALIN TEST
A
C
PITUITRIN TEST
C
P

DIAGRAM 2

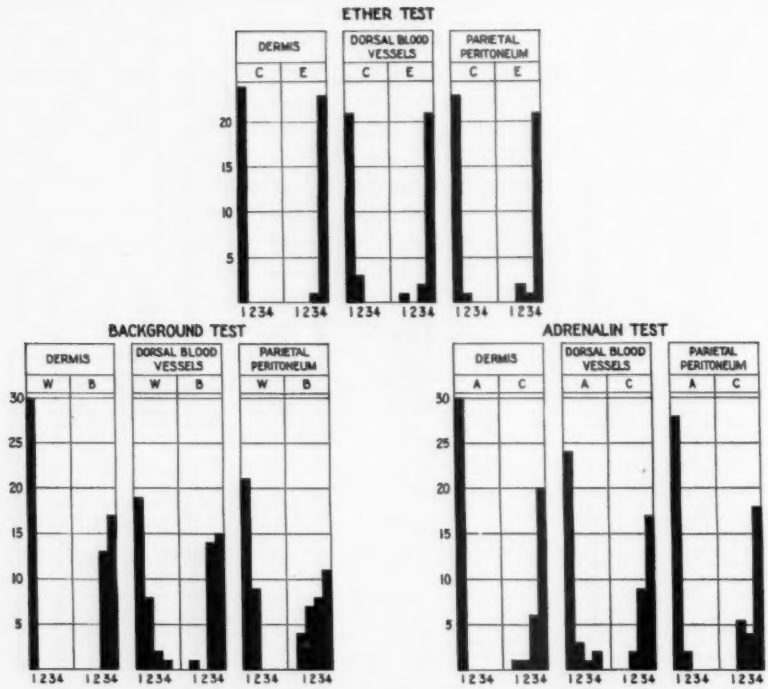


DIAGRAM 3. Tabulation of the effects of ether, background, and adrenalin on the melanophores in three different parts of the killifish: the dermis, the connective tissue covering of the dorsal blood vessels, and the parietal peritoneum (as shown at the top of each column).

In the ether tests, the fish with a few cubic centimeters of this drug added to the water fifteen minutes before their examination and their controls in water alone are distinguished in the diagram by *E* or *C* at the top of each column in the upper row.

In these tests, as in all subsequent ones, the results of the factor effecting the more complete contraction is placed at the left and that producing the less complete to the right in contrasting each pair.

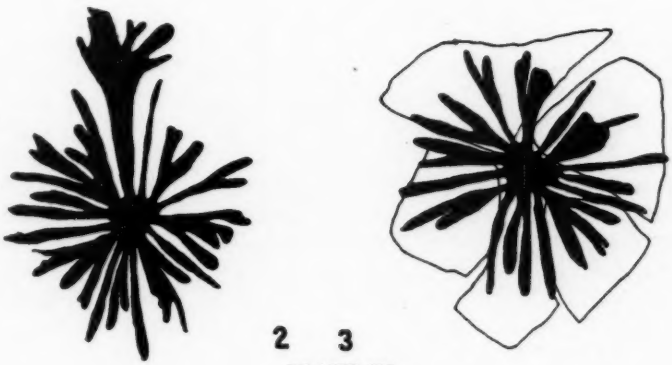
Forty-eight fish were kept in white dishes for a day before observation, of which but half were treated with ether. In every fish, the amount of melanophore contraction was noted as belonging to one of four stages of contraction of which 1 is maximum contraction and 4 minimum contraction. The number of cases falling in each class is recorded in black columns over the appropriate figure. Thus, in the dermis of fish kept on a white background, 24 showed complete contraction: on a white background with ether added to the water, 1 showed part expansion, and 23 complete expansion. All the remaining tests are tabulated in a similar fashion.

In the background tests, the shade of the surroundings, white or black, is indicated by *W* or *B* at the top of each column in the lower left-hand chart. Thirty fish were kept on each background for a day before observation.

In the adrenalin tests, both the fish treated with 0.2 cc. injection of the drug half an hour before examining and their controls with 0.2 cc. tap water injections are designated *A* or *C* respectively at the top of each column in the lower right chart. The 30 fish in each series were kept on an illuminated black background for a day before observation.

amphibia, those in *Fundulus* are scarce. Those in the mid-dorsal part of the parietal peritoneum and in the connective tissue of the dorsal blood vessels are large sheet-like cells. Those in the latero-dorsal portions of the parietal peritoneum and the more dorsal parts of the mesenteries and visceral peritoneum branch radially. In the visceral peritoneum, the true melanophores were usually obscured by melanin-filled syncytial cells—the " Pigmentflecken " of Ballowitz (1920). (Pl. IV.)

1



2 3

PLATE IV

Semidiagrammatic drawings of completely expanded melanophores of the killifish in (1) the connective tissue covering the dorsal blood vessels, (2) the parietal peritoneum, and (3) the visceral peritoneum (including the syncytial melanin-filled cells in outline).

All the melanophores started to contract when the fish were transferred to white surroundings and a day in them insured complete contraction in the majority of the cells (30 cases). Indeed, expansion of these indicated a slow dissection. Although not always completed for several days, there was an immediate distal migration of the pigment in all the melanophores with a change to the black jars (30 cases) (Diagram 3, lower left-hand part).

Even more rapid changes may be effected by injecting 0.2 cc. of adrenalin into the body cavity or tail muscles. Within half an hour the melanophores were well contracted (30 cases) and this condition persisted even after killing the fish, except in some of the cells in the connective tissue of the dorsal blood vessels (Diagram 3, lower right-hand part).

Pituitrin does not affect killifish melanophores. Hence, ether, which produces an expansion of the dermal melanophores (Wyman, 1924), was used instead. The addition of a few cubic centimeters to the water in which the fish were swimming over a white background, proved to exert the same effect on the internal melanophores (Diagram 3, upper part).

Discussion

The similarity in appearance of the deep-seated and dermal melanophores suggests the occurrence of the same activities in both sets of cells. Nevertheless, earlier work seemed to show that this was seldom the case. Yamamoto (1931), in working on the peritoneal melanophores of a Japanese minnow, obtained little, if any support for a nervous control of such cells. However, since the melanophores which he described and figured were quite different from those in the dermis, they were probably the " Pigmentflecken " studied by Ballowitz (1920) rather than true melanophores, since these are scarce, if not entirely lacking in the peritoneum of many species. These may also be the cells Gilson (1926) observed in adult *Fundulus*. In the newly-hatched larvæ, he noted responses in the true melanophores apparently before they were masked by the development of these syncytial cells, and came to the conclusion that they were under nervous control. Later, in full grown killifish, as the expanded state usually persisted in the internal melanophores despite changes in the external coloring of the fish, he considered that there was little evidence for such control. Thus it appears likely that he actually recorded the inactivity of the " Pigmentflecken." In adult *Fundulus* there are few melanophores in the more dorsal portions of the visceral peritoneum, and these are not easy to find except in fish deficient in pigmentation. For this reason, most of the observations in this report were confined to the melanophores of the parietal peritoneum. In this region, the cells were found to respond synchronously with those of the dermis. These findings, with Gilson's results on the larvæ, make it appear likely that the deep-seated melanophores of the killifish, like those in the skin, are chiefly under nervous control.

In amphibians the earlier work also furnished little evidence for any activity in the deep-seated melanophores, but for quite different

reasons. Lieben (1906) and Uyeno (1922) tried the effects of adrenalin by perfusing the tissues with it or by its injection a short time before killing the frog. In some cases they recorded a slight proximal migration of the melanin. It now appears that the cause of this apparent inactivity is due to the unusually slow response of these cells. In my experience, the deep-seated melanophores of frogs did not react for at least three hours and those of tadpoles for nearly five hours. The observations of Lieben and Uyeno were made on animals which had been killed before the application of the drug or soon after its injection. Hence there was time for disintegration of the melanophores to set in before they could have responded to the drug. Indeed, it was natural to suppose that, if the drug calls forth any response in the deep-seated melanophores, it would do so in about the same time as it does in the dermis. Yet this never happens. Does this slower response mean that the dermal and internal cells are not controlled by the same agencies? In view of the fact that all the melanophores of fishes are excited by the same means, it would be peculiar to find that the deep-seated and dermal melanophores of amphibians are activated along different pathways, especially as they all react more or less slowly. The dermal melanophores of animals belonging to this group, unlike those in fishes, are known to be predominantly influenced by humoral agencies (Hogben, 1924). If we assume a similar situation to exist among the deep-seated melanophores, what is the explanation of the delayed response in these cells? Since the activating materials are carried in the circulation, there might be differences in the blood supply, on the one hand, to the dermal and, on the other, to the deep-seated melanophores. Because the skin is an accessory respiratory organ, there is probably plenty of oxygen available there. On the contrary, within the animal, there may be less of it and more carbon dioxide. Some observations have been made on the diverse effects of these two gases (Lowe, 1917; Uyeno, 1922; Wyman, 1924). However, their action is seemingly not a factor, because in the end the responses of the two sets of melanophores are essentially similar. A more likely explanation may be found in the distribution of the blood vessels themselves in the respective parts. While the skin is ramified by a network of veins and arteries, the internal connective tissues are supplied by relatively few vessels. Thus, unlike the skin, the melanophores in the deep-seated portions of the animal are often more remote from the blood supply. Not only would it take longer for the humoral materials carried in the blood stream to reach most of the melanophores on account of the longer portage in the lymph system, but these would also become progressively diluted by the lymph the farther away their source in the blood. The former

situation may perhaps be the cause of the slower rate of response of the deep-seated melanophores. The latter, since the cells are not equidistant from the blood vessels, would account for the less extreme and more variable states of expansion found among them. In this way, the differences in the response of the dermal and internal melanophores may be explained. Accordingly, it is probable that the deep-seated melanophores are also controlled by humoral agencies.

The occurrence of similar activities in the dermal and deep-seated melanophores brings up the question of the function of these cells. Many representatives of the lower vertebrates by an expansion or contraction of the dermal melanophores, mimic, to some extent, the shade of their surroundings. This is often interpreted as being a protective measure or an aid in capturing their prey. If this be so, it seems odd, at first, that such changes should also take place within the animal. However, when we consider how sluggish the response is in the deep-seated melanophores of frogs and tadpoles, it is unlikely that the changes in the dermal cells are often reflected in the deeper portions of the animal. Accordingly, it is hard to believe that these cells by reason of their activities are ever of any particular use to the animal. In *Fundulus,* on the other hand, where the internal melanophores may be expected to react synchronously with every fleeting color change in the skin, there are so few true melanophores that it is difficult to conceive of their ever playing a very great rôle. Thus, either on account of their sluggish responses or their scarcity, the activities of the deep-seated melanophores are insignificant as compared with those in the skin. Hence, the fact that the deep-seated melanophores may display many of the activities of those in the skin does not make it necessary to dispense with the usual idea that the function of color change is an adaptive one.

Summary

In frogs, the deep-seated melanophores of the fascia of the leg muscles, of the muscles of the back, of the muscles of the body wall, of the mesenteries, and of the pleura respond to white or black backgrounds, adrenalin, and pituitrin in the same manner as do those in the skin, but about six times more slowly.

In tadpoles, the deep-seated melanophores of the fascia of the body-wall muscles, of the mesenteries, of the pericardium, and of the pleura respond to white or black backgrounds, adrenalin, and pituitrin in the same manner as do those in the skin, but react about ten times more slowly to the drugs.

In killifish, the deep-seated melanophores of the parietal peritoneum

and the connective tissues of the dorsal blood vessels respond to white or black backgrounds, adrenalin, and ether synchronously with those in the skin.

It is suggested that the difference in the speeds of response of the deep-seated melanophores in fishes and in amphibians is dependent upon the two methods of control which exist in the dermal melanophores of these two classes—nervous for fishes and humoral for amphibians.

In amphibians, the difference in the speed of dermal and deep-seated melanophores is believed to be due to the distribution of the blood vessels. While the skin is ramified with small blood vessels, the deep connective tissue receives a relatively scanty supply, for while many large veins and arteries pass through this tissue, they give off few branches to these parts. Also, the less extreme and more variable condition found among the deep-seated melanophores is thought to be caused by a dilution of the activating materials by the lymph.

The similarity of response in the deep-seated and dermal melanophores does not reveal what function these cells may serve.

Although the internal melanophores obviously play no part in color changes, neither does their activity interfere with the usual idea that the function of color changes in the skin is an adaptive one, because they are either so few in number that their rôle must be slight (fishes), or so sluggish that they seldom reflect the changes of the skin (amphibians).

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THE CHROMOSOMES OF HABROBRACON

MAGNHILD TORVIK-GREB

(From the University of Pittsburgh, Pittsburgh, Pennsylvania)

The parasitic wasp *Habrobracon juglandis* (Ashmead) has, for some years, been a subject of genetic investigations. These investigations have consistently demonstrated that in *Habrobracon,* as in Hymenoptera in general, females come from fertilized eggs and are diploid while males come from unfertilized eggs and are haploid. Occasionally biparental males are produced that inherit from the father as well as from the mother. Genetic evidence indicates that they are diploid and that their daughters are triploid. Rarely, and only in a few stocks, daughters are produced by virgin females.

In 1918 P. W. Whiting made a preliminary cytological study of this wasp. He found that the males were haploid, that the first meiotic division was abortive and that the second was equal. Later Anna R. Whiting (1927) gave a tentative chromosome count of 11 for the male and 22 for the female. She further stated that, " in spermatogenesis of biparental males the first maturation division is abortive, the second apparently equational as in normal haploid males."

Since *Habrobracon* proved to be difficult material for cytological study, and since preliminary work indicated chromosome behavior to be comparable to that of many other Hymenoptera, the work was abandoned temporarily.

Meves, as early as 1904, discussed spermatogenesis in the wasp, *Vespa germanica,* showing the abortive first meiotic division and the equal second division characteristic in spermatogenesis of haploid males of many Hymenoptera.

The cytology of parthenogenetic forms was thoroughly reviewed and a bibliography given by A. Vandel in 1931. In the same year Franz Schrader and Sally Hughes-Schrader published a discussion of " Haploidy in Metazoa " in which they included a summary of haploid forms studied cytologically and an extensive bibliography. More recently (1933) Ann R. Sanderson published a critique of the literature and a bibliography of the cytology of parthenogenesis in Hymenoptera.

Although *Habrobracon* is unfavorable material, it has become increasingly necessary to undertake a cytological study since there are many aberrant types known (diploid males, triploid females and daughters of virgin females as well as male mosaics and gynandromorphs).

The present paper deals with the chromosomes of normal males and females as well as with those of biparental males.

Material and Methods

Gonads of freshly eclosed males and females were used for this study. Early in the work it was thought that male gonads of larvæ or pupæ might show more division figures and a few of these were examined. This material proved to be less favorable than that from adult males. Females were allowed to feed on caterpillars, which they normally parasitize, for a few days before dissection in order to insure their being in an active egg-laying condition.

Wasps were dissected in water, gonads removed and immediately

EXPLANATION OF PLATES

A Zeiss camera lucida and a Spencer microscope equipped with a Zeiss K30X ocular and a Zeiss oil immersion apochromatic 1.5 mm. objective were used in making the drawings. The original magnification of the drawings was 6,900 diameters, but these were enlarged to 13,800. They have been reduced to one-third in reproduction giving a final magnification of 4,600 diameters.

The chromosome complexes shown in polar views of metaphases in Plates I–III are repeated in Plate IV with chromosomes arranged in decreasing order of magnitude for comparative study. These polar views are from sixteen specimens with drawings corresponding as follows:

Specimen number	Drawings	
	Plates I–III	Plate IV
1	5	A 2
2	6	A 1
3	7, 8	A 4, 3
4	15, 16, 17	B 6, 7, 8
5	18	B 3
6	19	B 2
7	20	B 1
8	21	B 4
9	22	B 5
10	27	C 3
11	28, 29	C 1, 2
12	34	D 2
13	35	D 1
14	36	D 3
15	38	E 1
16	39, 40	E 2

Explanation of Plate I

Figs. 1–14 from normal haploid males.

Figs. 1–2. Early spermatogonia.

Fig. 3. Later spermatogonia.

Fig. 4. Spermatogonial metaphase, side view.

Figs. 5–8. Spermatogonial metaphases, polar views.

Figs. 9–10. Spermatogonial anaphases, side views.

Figs. 11–13. First spermatocytes. Views of the abortive first meiotic division showing the cytoplasmic bud being pinched off.

Fig. 14. Second spermatocyte metaphase, side view.

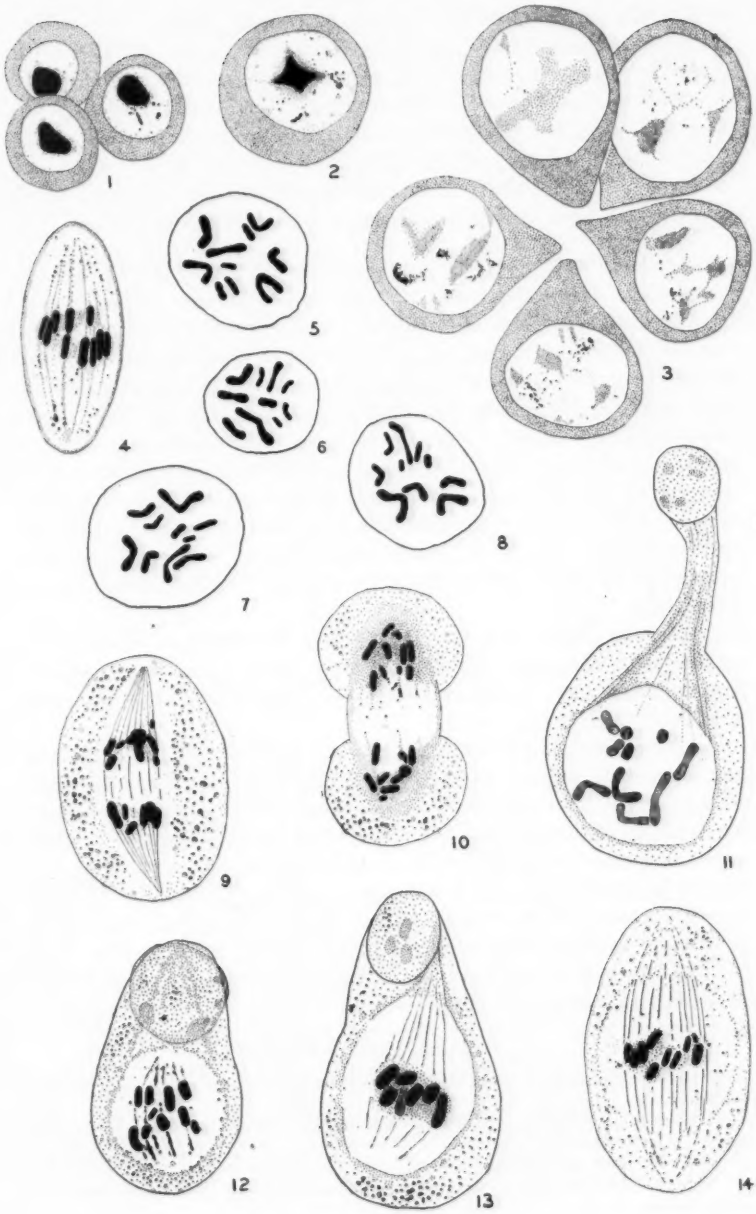

PLATE I

transferred to the fixative. Eleanor Carothers' method for orthoptheran cells was followed in fixing (McClung, C. E., 1929, p. 186). After having been washed and passed through the lower alcohols, gonads were stained *in toto* with eosin in 95 per cent alcohol to guard against possible loss in later handling. They were run through aniline oil and chloroform into paraffin. Sections were cut five to six microns in thickness. Heidenhain's iron hæmatoxylin was used for staining all slides studied—the short method being preferred to the long.

Chromosomes of the Normal Haploid Male

In *Habrobracon* the paired, rounded, yellowish testes (about ¼ mm. in diameter) are found fused dorsal to the intestine in the posterior part of the body. Each gives off a lateral duct which passes around the intestine and unites with the accessory gland to form a common duct which connects with the penis. Internally the testes are subdivided into a number of cysts. Scattered throughout the testes of immature individuals are spermatogonial cells many of which are in compact rosettes but some are in larger, looser rosettes. In the gonads of late pupæ maturation division figures were observed and even some spermatids, but gonads of freshly eclosed males showed the highest percentage of cells in active division and were, therefore, studied most intensively.

No attempt was made to follow closely the course of development of the spermatogonia. Rosettes of very small cells were observed, growth stages were noted and rosettes of fully-grown cells also were recorded (Plate I, Figs. 1–3).

Good mitotic figures of spermatogonia were rarely seen. In any one testis there was never more than one cyst showing spermatogonial mitoses and in most testes no such divisions were in progress. However, enough figures (from ten different wasps) were found to offer convincing proof of the fact that normally there are ten chromosomes present in spermatogonial cell divisions of the haploid male. The four spermatogonial metaphase plates shown (Plate I, Figs. 5–8) were taken from testes of three different wasps. Counts were made in other cases some of which were below ten, but in such instances plates were slanted or obscured by other cells in the section.

Explanation of Plate II

Figs. 15–22. Second spermatocyte metaphases, polar views, from normal haploid males.

Figs. 23–25. Second spermatocyte anaphase and telophases, side views, from normal haploid males.

Fig. 26. Late spermatogonia from biparental diploid male.

Figs. 27–29. Spermatogonial metaphases, polar views, from biparental diploid males.

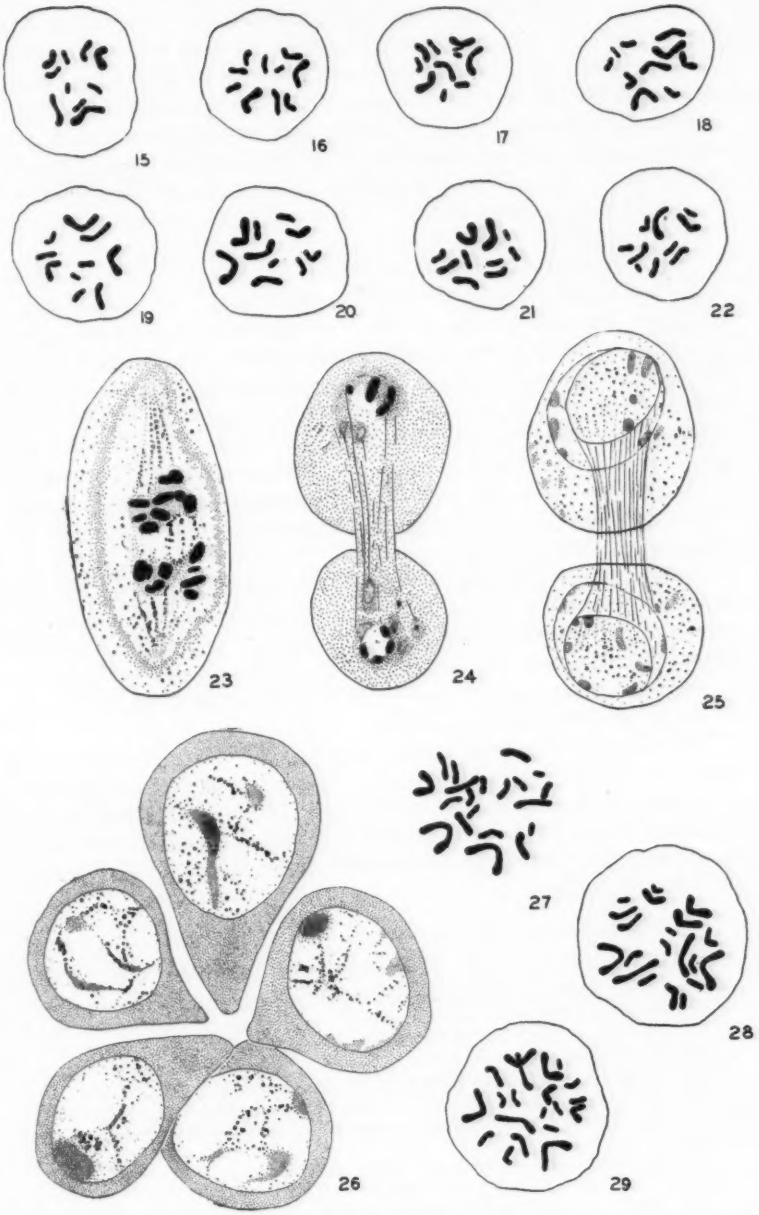

PLATE II

In later prophase stages of the first spermatocyte, after the nucleus has increased in size, the chromatin appears as small bodies lying close to the nuclear wall. Still later the cells are pear-shaped in preparation for the abortive first meiotic division. The chromatin bodies, no doubt condensing chromatin threads, develop into chromosomes which first appear near the nuclear wall and then condense as if in preparation for the division. Meanwhile, a small bud has been cut off at the narrow end of the pear-shaped cell. This bud, which is purely cytoplasmic, soon degenerates (Plate I, Figs. 11–13).

The chromosomes then lengthen into threads but soon condense again and travel to the center of the nuclear mass. The nuclear membrane has now disappeared and the chromosomes remain at the center of the cell where they immediately become aligned on a metaphase plate for the second meiotic division. Among fifty-seven drawings of such plates; forty-seven showed ten chromosomes, seven showed nine, while three had apparently eleven chromosomes. Plates of the two latter groups had questionable regions as did some of the others. However, the best plates consistently showed ten chromosomes. The eight shown in the drawings (Plate II, Figs. 15–22) were taken from sections of testes of six different wasps.

In the second spermatocyte division the cytoplasm divides equally and each chromosome divides. The chromosomes can be counted in some of the anaphase stages but in telophase they are too closely clumped for counting. Following division a nuclear membrane appears, the chromatin opens out and becomes dispersed preceding the formation of the sperm (Plate II, Figs. 23–25).

The stages in transformation of the spermatid were not followed in detail. In an early stage the chromatin appears near the inner surface of the nuclear membrane, first as large granules, later as a heavy band. Elongation of head and tail takes place until finally bundles of spermatozoa are seen, their heads together and their tails usually pointing towards the center of the cyst.

Chromosomes of the Biparental Diploid Male

It was first noted in 1921 (Whiting, P. W., 1921) that occasionally males in *Habrobracon* show paternal traits and, therefore, do not arise

Explanation of Plate III

Figs. 30–37 from biparental diploid males.

Figs. 30–31. Spermatogonial metaphases, side views.

Figs. 32–33. First spermatocytes. Views of the abortive first meiotic division, showing the cytoplasmic bud being pinched off and the diploid chromosome complex.

Figs. 34–36. Second spermatocyte metaphases, polar views.

Fig. 37. Second spermatocyte telophase.

Figs. 38–40. Oögonial metaphases, polar views, from normal diploid females. Figs. 39 and 40 are from sections of a single cell.

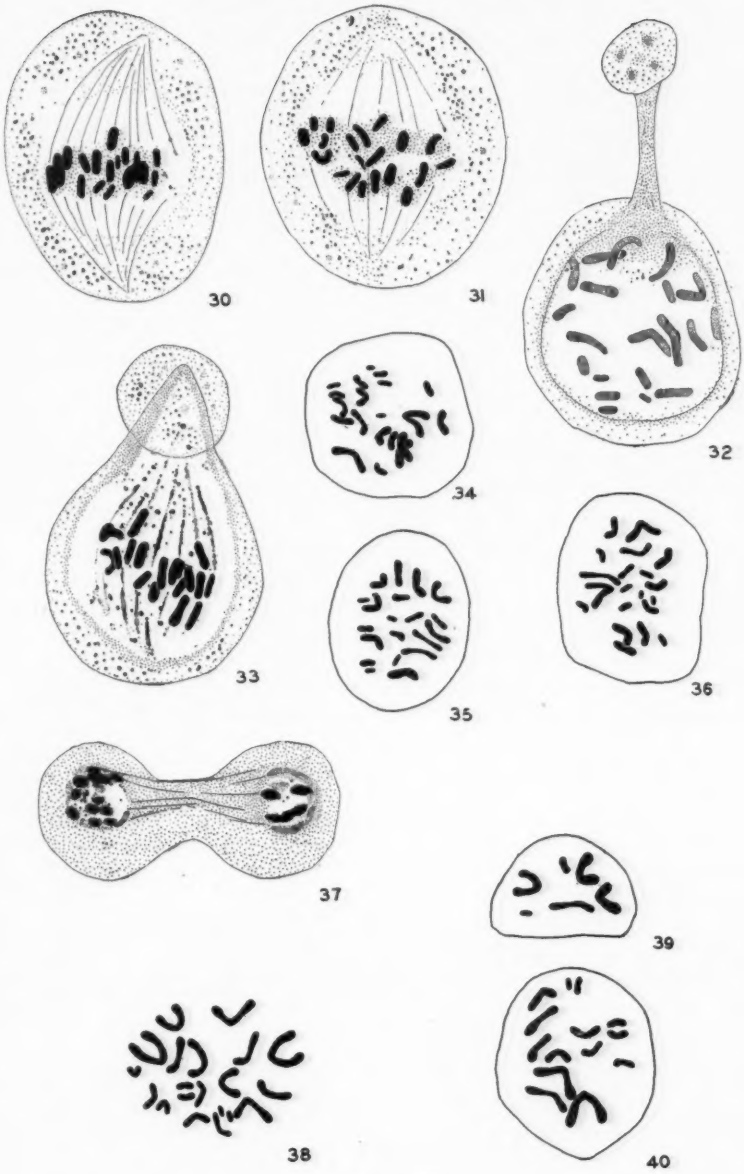

PLATE III

from unfertilized eggs. Subsequent genetic tests have shown that these males occur when related stocks are crossed (Whiting, Anna R., 1925) and that they are diploid (Whiting, Anna R., 1927; Torvik, Magnhild M., 1931).

Cytological evidence of the fact that these males are diploid is now at hand. Cytological observations also show that spermatogenesis follows the same course in the diploid male as in the haploid male. The cells are necessarily larger throughout to accommodate the greater amount of chromatin present but cell growth and cell division proceed similarly in both cases. The figures shown in Plates II and III present some of the more important stages.

Three metaphase plates of spermatogonial divisions (from two wasps) are included (Plate II, Figs. 27–29). These have twenty easily distinguishable chromosomes. There are also three metaphase plates of the second spermatocyte (from three other wasps). Again twenty chromosomes are apparent (Plate III, Figs. 34–36). Among twenty metaphase plates drawn the seven clearest (from six wasps) had twenty chromosomes each; five showed nineteen; four showed eighteen; and four had apparently more than twenty chromosomes. The last no doubt were entering anaphase while the plates giving low counts probably had chromosomes overlying one another in ways which made them indistinguishable. Homologous chromosomes exhibited no great tendency to lie in pairs. By chance they would occasionally lie close to one another. The first meiotic division is abortive and the second apparently equal. Consequently, the sperm formed would be at least approximately diploid, as indicated by genetic tests.

Chromosomes of the Normal Diploid Female

The paired gonads of *Habrobracon* females each consist of two ovarioles morphologically made up of three regions: (1) dorsal, apical end-chamber with oögonia, followed by (2) masses of nurse cells alternating with (3) developing oöcytes each of which is surrounded by follicle cells. Each ovary ends in an enlarged sac or uterus in which mature eggs are contained.

Sections were made of entire ovaries but only oögonial division figures were noted. Metaphase plates were found in ovaries from seven different females. Among ten plates which were drawn six had twenty chromosomes each. The remainder were less clear and, therefore, more difficult to count correctly. Two oögonial metaphase plates are shown (Plate III, Figs. 38–40).

Chromosome Comparisons

The chromosomes of *Habrobracon,* though small, do exhibit differences with respect to size and shape. This was most evident in meta-

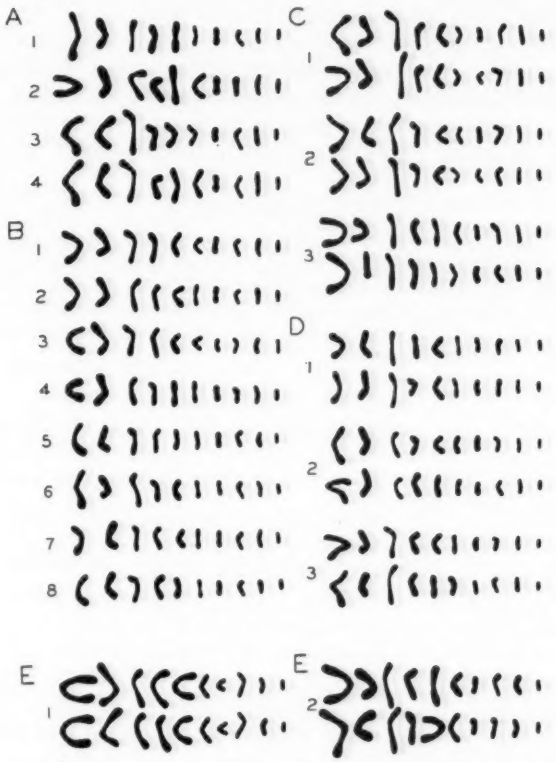

Plate IV

Metaphase chromosome complexes from Plates I–III. From left to right there are shown in decreasing order of magnitude: one *V*, one *L*, one *J*, one *L*, three *V*'s, one *J*, one which is often rod-like but may be a *V*, one rod.

Figs. *A*, 1–4. Spermatogonial complexes of haploid males.
Figs. *B*, 1–8. Second spermatocyte complexes of haploid males.
Figs. *C*, 1–3. Spermatogonial complexes of diploid males.
Figs. *D*, 1–3. Second spermatocyte complexes of diploid males.
Figs. *E*, 1–2. Oögonial complexes of diploid females.

phase plates. In order to make comparisons easier, the chromosomes already shown in views of metaphase plates have been arranged linearly in Plate IV, according to size. In the haploid male there appear to be:

four *V*'s, one of which is always large, two medium to large *L*'s, two *J*'s, one small rod and one chromosome which often is rod-like but may be a small *V*. A study of these figures convinces one of the fact that the diploid group consists of pairs of chromosomes whereas the haploid group contains but one chromosome of each type.

It is interesting to note that the oögonial chromosomes are the largest of those studied. Next in size are the spermatogonial chromosomes of both haploid and diploid males. Spermatocyte chromosomes are the smallest. While they vary somewhat in size, they tend to be thicker and more condensed than other chromosomes observed.

ACKNOWLEDGMENTS

The writer is indebted to Professor P. W. Whiting for suggesting the problem and for guidance. Professor Robert T. Hance generously provided laboratory facilities and help. Dr. G. M. McKinley deserves special mention for aid in obtaining satisfactory lighting facilities. The Committee on Effects of Radiation (National Research Council) has furnished technical assistance and apparatus by grants to Professor Whiting.

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COMBINATIONS OF CURRENT AND ANTECEDENT CONDITIONS IN RELATION TO WING-PRODUCTION OF APHIDS[1]

A. FRANKLIN SHULL

(*From the Zoölogical Laboratory of the University of Michigan*)

The influence of environmental conditions and genetic or other internal factors upon wing-production in the aphid *Macrosiphum solanifolii* has been repeatedly demonstrated in controlled experiments (Shull, 1928, 1929, 1932). For the most part the environmental factors have been tested singly, while all other agents were kept as nearly constant as possible. This was the best way to demonstrate that a given agent has an influence. In the course of the experiments, however, it became evident that the effect of one factor might easily depend upon the accompanying conditions. It was not merely that the factors often worked in opposite directions, so that their combined effect would be the algebraic sum of their single effects; it was found that the action of one agent might change, not merely in amount but even in sign, in response to changes in other factors.

It became desirable, therefore, to use the several agents in their various combinations. The experiments here described constitute the first exploration of the possible modifying, accentuating, and inhibiting effects. Light, temperature, and the presence or absence of wings in the parent aphids are the chief factors so far tested; and since it would be impossible with the facilities available to use all the known modifying agents, it was decided to use only these three. As a further means of curtailing the labor, only two conditions in each of these fields were employed. The two conditions chosen were such as were known to have different effects, and as would permit a not too slow accumulation of data. With respect to light, the two conditions were continuous light and alternating light and darkness (eight hours of the former, sixteen hours of the latter). As to temperature, 24° and 14° C. were selected; temperatures outside of this range are apt to have deleterious effects. The nature of the parent aphids fell into the two classes, wingless and winged, though it would have been possible and desirable to use intermediate-winged individuals as well.

To these three groups of conditions there was reason to suspect a fourth should be added. This was the set of conditions under which

[1] This work has been aided by a grant from the National Research Council and the Elizabeth Thompson Science Fund.

the stock of aphids was living from which the parent aphids were drawn for the experiments. While it was desirable that various sets of "stock" conditions be tested (involving light, temperature and wings), no more than one of these could be used within the limits of time and space; and temperature was selected as the condition to be varied in the stocks. Accordingly, more than three years ago one stock was started at 24° C., another at 14° C., both in continuous electric light. Later a third stock was kept at alternating temperatures, 24° during eight daytime hours, 14° for sixteen hours at night. Continuous electric light has been furnished for all these stocks, and daylight was practically excluded. One of the stocks was killed by the breakdown of the constant temperature apparatus, but was at once replaced and soon appeared to give results identical with those of the original stock.

The experiments have started with aphids from each of these three stocks. Some of the aphids from each stock were winged, others wingless. Each kind (winged and wingless) was divided into two groups, one raised at 24°, the other at 14°. At each of these temperatures, some were given continuous light, others alternating light and darkness (eight hours light, sixteen hours darkness). Twenty-four groups of parent aphids were thus necessary for a complete experiment. For some of these groups the conditions under which they were reared represented merely a continuation of their former conditions. For others, they represented a change in temperature, or in light, or in both temperature and light. Only the parents were subjected to the conditions named. Their offspring were brought, in 2-day or 4- or 5-day batches depending on temperature, to room conditions to complete their growth.

Owing to the erratic fluctuations to which wing-production in nearly all aphids seems to be subject, a single experiment of the sort just outlined could not be expected to give reliable results. The experiments have therefore been many times repeated. In this paper are recorded over 167,000 aphids. The rearing of such large numbers has been made possible only by the watchfulness and meticulous exactness of Dr. Helen F. Price; without her aid the experiments must have failed long ago. Over long periods of time the repetitions of the experiments have tended to give constant results; that is, in nearly every test there have been the same kinds of differences between the various groups of offspring. This degree of uniformity in the general results engenders confidence that the contrasts shown by the totals do actually represent the effects of the various combinations tested.

Results of the Experiments

While it would be instructive to give the results of the several repetitions of each test, or even the daily output of each group of parents, in

TABLE I

The number of winged and wingless offspring from parents derived from certain stocks and reared under certain conditions. Data are arranged to show most directly the influence of light.

Parents				Offspring		
Winged or wingless	From stock reared at temperature	Conditions under which reared		Wingless	Winged	Percentage winged
		Temp. ° C.	Light			
Wingless	24°	24	Cont.	3657	3662	50.0
			8–16	3722	1798	32.6
		14	Cont.	4021	3509	46.6
			8–16	4242	881	17.2
	14°	24	Cont.	3309	3315	50.0
			8–16	2474	2482	50.1
		14	Cont.	3207	3952	55.2
			8–16	3750	1081	22.4
	Alt.	24	Cont.	2105	2405	53.3
			8–16	2569	1954	43.2
		14	Cont.	3996	2384	37.4
			8–16	4037	667	14.2
Winged	24°	24	Cont.	4716	7002	59.6
			8–16	3678	2428	39.8
		14	Cont.	9358	7127	43.2
			8–16	12465	3208	20.5
	14°	24	Cont.	5226	2747	34.5
			8–16	5140	1422	21.7
		14	Cont.	5162	1384	21.1
			8–16	2990	439	12.8
	Alt.	24	Cont.	2585	3682	58.8
			8–16	2635	2864	52.1
		14	Cont.	4487	3131	41.1
			8–16	4134	598	12.6
Total				103665	64122	

order to show the fluctuations referred to in the preceding section, space forbids any such detailed presentation. The total numbers are large enough, it is believed, to insure that the fluctuations largely neutralize

one another. The data have not been given statistical treatment because it is uncertain what the real unit of expression is. The validity of the contrasts shown seems to be assured by the fact that in most in-

TABLE II

The same data as those given in Table I, arranged to show most directly the influence of the temperature at which the parents were reared.

Parents				Offspring		
Reared in light	Winged or wingless	From stock reared at temperature	Reared at temp.	Wingless	Winged	Percentage winged
Cont.	Wingless	24°	24°	3657	3662	50.0
			14°	4021	3509	46.6
		14°	24°	3309	3315	50.0
			14°	3207	3952	55.2
		Alt.	24°	2105	2405	53.3
			14°	3996	2384	37.4
	Winged	24°	24°	4716	7002	59.6
			14°	9358	7127	43.2
		14°	24°	5226	2747	34.5
			14°	5162	1384	21.1
		Alt.	24°	2585	3682	58.8
			14°	4487	3131	41.1
8–16 hr.	Wingless	24°	24°	3722	1798	32.6
			14°	4242	881	17.2
		14°	24°	2474	2482	50.1
			14°	3750	1081	22.4
		Alt.	24°	2569	1954	43.2
			14°	4037	667	14.2
	Winged	24°	24°	3678	2428	39.8
			14°	12465	3208	20.5
		14°	24°	5140	1422	21.7
			14°	2990	439	12.8
		Alt.	24°	2635	2864	52.1
			14°	4134	598	12.6

stances the experiments can be grouped, all or most of the experiments in one group showing the same type of contrast. These facts will be fairly apparent on inspection of the data.

The total numbers of winged and wingless offspring born of parents derived from the three stocks and reared under different conditions are shown in Table I. As there arranged, it is indicated that wingless and winged parents were taken from each of the three temperature stocks (24°, 14°, alternating); that of each group some were reared at 24°, others at 14°; and that at each temperature some were reared in continuous light, others in alternating light and darkness (eight and sixteen hours, respectively). The offspring from these twenty-four sources are given at the right, with the percentage of winged individuals among them.

Contrast of Light Conditions Under Which Parents Were Reared

The arrangement of the data after any scheme similar to that in Table I is best fitted to contrast the effect of the conditions indicated in the fourth column of the branching tree. In Table I this contrast is between continuous light and alternating light and darkness. Pair by pair the numbers to the right of this column, more particularly the percentages in the last column of the table, show the different results from these two light conditions.

In every pair of experiments except one, regardless of how they differed in other respects, more winged offspring were produced in continuous light than in alternating light and darkness. In the one exceptional pair, the third in the table, the two light treatments had practically identical effects. In one other pair (the eleventh) there would be room to question the significance of the difference if it stood alone. But with every pair excepting one showing a difference of the same sign, and most of them a difference of considerable size, there can be but one conclusion: continuous light in general favors wing-production in this strain of aphids, as against alternating light and darkness.

Contrast of Temperatures at Which Parents Were Reared

The results of the same experiments are arranged in Table II with the temperatures at which the parents were reared placed in the fourth column. This position facilitates comparison of the effects of these two temperatures, since each pair of percentages in the last column of the table shows that contrast directly for one combination of the other factors.

A glance at the right column shows that all of the pairs of percentages show differences of the same sign except one. The first two differences, which include the exceptional one and another which agrees with the majority in sign, are small and are perhaps not significant. The

rest all indicate that distinctly more winged offspring are produced at high temperature (24°) than at low (14°), no matter what other conditions are combined with it.

TABLE III

The data of Table I rearranged to show most directly the effect of wings or their absence in the parents upon wing-production in their offspring.

Parents				Offspring		
From stock reared at temperature	Reared at temp.	Reared in light	Winged or wingless	Wingless	Winged	Percentage winged
24°	24°	Cont.	Wingless	3657	3662	50.0
			Winged	4716	7002	59.6
		8–16	Wingless	3722	1798	32.6
			Winged	3678	2428	39.8
	14°	Cont.	Wingless	4021	3509	46.6
			Winged	9358	7127	43.2
		8–16	Wingless	4242	881	17.2
			Winged	12465	3208	20.5
14°	24°	Cont.	Wingless	3309	3315	50.0
			Winged	5226	2747	34.5
		8–16	Wingless	2474	2482	50.1
			Winged	5140	1422	21.7
	14°	Cont.	Wingless	3207	3952	55.2
			Winged	5162	1384	21.1
		8–16	Wingless	3750	1081	22.4
			Winged	2990	439	12.8
Alt.	24°	Cont.	Wingless	2105	2405	53.3
			Winged	2585	3682	58.8
		8–16	Wingless	2569	1954	43.2
			Winged	2635	2864	52.1
	14°	Cont.	Wingless	3996	2384	37.4
			Winged	4487	3131	41.1
		8–16	Wingless	4037	667	14.2
			Winged	4134	598	12.6

If, despite their smallness, the differences in the first two pairs are significant, the presence or absence of a *change* in the temperature may be responsible for influencing wing production. In the first pair, those

parents which came from a stock reared at 24° and were continued at 24° in the experiments produced the larger number of winged offspring. In the second pair those parents which came from a 14° stock and were continued at 14° produced the more winged offspring. It is possible that mere change from one temperature to another, whether from high to low or from low to high, reduced wing-production somewhat. In any case this could be said only of the wingless parents kept in continuous light. It is difficult to attribute any such effect to mere change in any other part of the table.

Contrast of Winged with Wingless Parents

The data of the experiments are rearranged in Table III in such a way as to show most plainly the effect of wings or their absence in the parents upon wing production in the offspring. This is done by placing the nature of the parents in the fourth column so that the pairs of percentages in the last column will show that particular contrast.

The most striking fact brought out by this arrangement is that the winged parents produced notably fewer winged offspring than did the wingless parents, provided the parents had been taken from the low temperature (14°) stock—but under no other circumstances. It made no difference in what light or temperature they were reared; if only they came from the 14° stock, the winged parents yielded the fewer winged offspring.

When the parents came from the 24° or the alternating temperature stocks, it made less difference whether they were winged or not. Indeed, it might be questioned whether wings made any difference in the offspring. Of the eight contrasts from these two stocks shown in the last column, the winged parents yielded the more winged offspring in six, and fewer winged offspring in two. One of the differences is 9.6 per cent, one 8.9 per cent, a third 7.2, the others less. While it seems likely that this preponderance of the results must indicate that in general wings in the parents favor wings in the offspring when the parents come from high or alternating temperature, the influence can only be slight and is presumably modified by some other factor.

Contrast of Temperatures from Which Parents Were Taken

By placing the temperature conditions of the three stocks in the fourth column the effects of these antecedents are most clearly shown. This is done in Table IV. In that table it is shown, so far as concerns the stocks reared at 24° and 14°, that in every instance more winged offspring were produced by parents taken from the 24° stock than by those taken from the 14° stock provided the parents chosen were winged.

The differences all seem certainly significant. But if wingless females were chosen as parents, then in general more winged offspring were produced by those coming from the 14° stock. There is one exception to this latter statement, namely, the first trio of percentages, in which the

TABLE IV

The data of Table I so arranged as to show most directly the effect of the temperature at which the parents and their ancestors were reared (prior to the beginning of experiments) upon wing-production in their offspring, with special reference to the 24° and 14° stocks.

Parents				Offspring		
Winged or wingless	Reared in light	Reared at temperature	From stock reared at temperature	Wingless	Winged	Percentage winged
Wingless	Cont.	24°	24°	3657	3662	50.0
			14°	3309	3315	50.0
			Alt.	2105	2405	53.3
		14°	24°	4021	3509	46.6
			14°	3207	3952	55.2
			Alt.	3996	2384	37.4
	8–16	24°	24°	3722	1798	32.6
			14°	2474	2482	50.1
			Alt.	2569	1954	43.2
		14°	24°	4242	881	17.2
			14°	3750	1081	22.4
			Alt.	4037	667	14.2
Winged	Cont.	24°	24°	4716	7002	59.6
			14°	5226	2747	34.5
			Alt.	2585	3682	58.8
		14°	24°	9358	7127	43.2
			14°	5162	1384	21.1
			Alt.	4487	3131	41.1
	8–16	24°	24°	3678	2428	39.8
			14°	5140	1422	21.7
			Alt.	2635	2864	52.1
		14°	24°	12465	3208	20.5
			14°	2990	439	12.8
			Alt.	4134	598	12.6

wing-production was practically identical for parents taken from both the 24° and 14° stocks.

It is difficult to see any relation between the experiments performed with parents from the alternating stock and those derived from either

of the other two stocks. In some experiments such parents produced more winged offspring than did those from either of the constant temperatures, in other experiments fewer than either, and in still others a number intermediate between those from the two constant temperatures. There appears to be no general rule stating these relations.

Distribution of Wing Production Through the Family with Respect to Age

It is important in judging the effects of different agents on wing production to know how the wings are distributed among the successive offspring of the treated parents. The experiments were so conducted as to make this information available. In all experiments the parents were removed to the stipulated conditions while in the late fourth instar or just after becoming adult. Their offspring were obtained in successive groups by changing the parents to a new plant every two days if at high temperature, or every four or five days (five in early experiments, four later) if at low temperature. Most families were practically complete in six to eight such successive groups. It is possible, therefore, to ascertain how the proportion of winged offspring changed from the beginning to the end of the family. It would again be instructive to show the families separately, but space forbids. All families derived from a common source and treated in the same way are collected into one lot, just as was done with the data so far presented.

The percentage of winged offspring from the twenty-four lots of parents is shown in the twenty-four curves of Plate I. To save tabular matter the actual numbers of individuals are not given. The total number represented by each curve may be ascertained from Table I. However, the number in each of the six to eight successive lots of offspring does not appear. In general, the early offspring were much more numerous than late ones; the last lot was sometimes so small that a percentage based on it could not be very reliable.

Effect of Age

For some of the groups of parents the conditions of temperature and light at which they were reared represented no change whatever. This is true of curves *A, D, N* and *Q,* which are darkened for ease of selection. Whatever change takes place in the percentage of winged offspring from one of these groups of parents may be looked upon as in some respect an effect of age. It is of interest to find that the change with age is of the same sort in all of them; the winged offspring become irregularly less numerous the older the parents are. The decline of the winged individuals is most rapid among the offspring of wingless parents

24°-Continuous Light
24°-Alternating Light and Darkness
14°-Continuous Light
14°-Alternating Light and Darkness
Percentage Winged
Winglessness From 24°-Stock
Winglessness From 14°-Stock
Winglessness From Alternating Temperature Stock
A
B
C
G
H
I
M
N
O
S
T
U

PLATE I

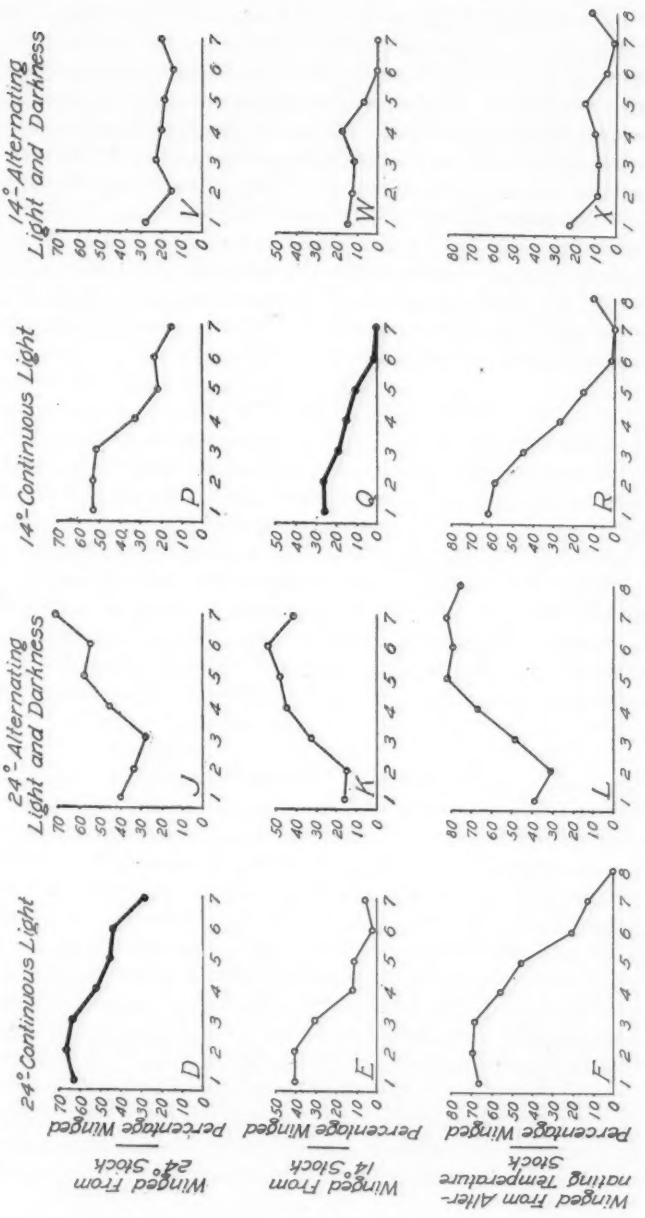

PLATE I—*Continued*

at 24° and continuous light (curve *A*). It is slowest, but unmistakable, among those from winged parents at either 24° or 14° and continuous light (curves *D* and *Q*); however, curve *D* is throughout at a much higher level than *Q*. Whether the decline in curve *N* is greater than that in *A* may be questioned. Except at the beginning, curve *N* is everywhere higher than *A*, but it can hardly be said that the difference increases from left to right; and the sharp rise at the end of *N* is based on only thirty-three offspring in the last 4-day output.

The decline in all these curves, representing an age effect, must of course be taken into account in judging the influence of the other factors. No effect of any other factor is to be inferred unless this result of age is accentuated or reversed or partially nullified or in some way modified in at least part of the family.

Effect of Changed Conditions

All curves other than *A*, *D*, *N* and *Q* represent the distribution of winged offspring through the family as affected by one or more changes of conditions. The first column of curves (*A*—*F*) show the slightest change of any in the entire set of experiments. Curves *B* and *C* do not differ in any striking way from *A*, which means that changing the parents from 14° or alternating temperature to 24° does not modify the distribution of winged offspring as determined by mere age. With respect to winged parents, curve *E* differs from *D* chiefly in being lower; its decline is about the same as that of *E*. The low level of wing-production in *E* is not, however, due to a change of the parents from 14° to 24°, since curve *Q* is even lower. Change from 14° to 24° sufficed to raise curve *E* somewhat, but not nearly so high as *D*. This presumably means that the temperature at which the parents were reared before the experiments began influenced the amount of wing production in their offspring more than did the temperature maintained during the experiments—a statement which applies only to winged parents. Curve *F* differs from *D* only toward the end of the family, where it shows sharply less wing-production than in *D*.

The curves in the second column of Plate I have one striking feature in common, namely, a sharp dip in wing-production about the first one-fourth or one-third of the family. This dip is quite marked when the parents are wingless (curves *G*—*I*), since the initial wing-production, as in most other families from wingless parents, is high. When the parents are winged (curves *J*—*L*) the initial wing-production is quite low, and the dip is less conspicuous or may even disappear. After this depression the curve rises sharply, leveling off or even declining at the end in some. No early depression occurs in any of the curves of the

first column (*A*—*F*); in fact, there is a slight tendency in these curves for the second lot of offspring to cause a hump in the curve, either by including more winged individuals than the first lot does, or by receding less than the later curve as a whole does. These depressions must therefore be attributed to the one factor which is different in curves *G*—*L* as contrasted with *A*—*F*, namely, the alternating light and darkness to which the parents were exposed during the experiment.

Curves *M*—*R*, obtained under a single set of conditions, but from different kinds of parents whose antecedent treatment was various, are not strikingly different. They all show a decline that is mostly attributable to age. There are no constant humps nor depressions. They start high when the parents are wingless, or when the parents are winged and raised previously in alternating temperatures. Their general similarity presumably means that 14° and continuous light during the reproductive period of the parents are more influential than any change to those conditions from any antecedent conditions. The low start of curves *P* and *Q*, particularly the latter, seems to be due to the fact that the parents were winged, though winged parents previously kept in alternating temperatures (curve *R*) did not start out with a small percentage of winged offspring.

Curves *S*—*X* in the last column of Plate I are very similar. Each presents at the outset a decline which is precipitous if the starting point was high (*S*—*U*), but moderate if the percentage of winged offspring was at first low (*V*—*X*). After this decline there is a moderate rise in wing-production, the peak of which comes at various places in the family. Following the rise there is another decline.

Effect of Intermittent Light

The outstanding general result of the various sets of conditions is the effect of alternating light and darkness during the experiments. The second and fourth columns of curves (*G*—*L* and *S*—*X*) in Plate I show this effect. A reduction of wing-production in the early part of the family followed by a rise later occurs in every one of these curves. The depth of the early depression depends chiefly on the initial amount of wing-production (which in turn depends chiefly on the presence or absence of wings in the parents), while the height of the subsequent rise depends mostly on the current temperature (high temperature increasing the rise). At high temperature there is little indication of a second fall after the rise, but at low temperature such a decline is present in every instance. It may be plausibly suggested that, if reproduction continued longer at high temperature (*G*—*L*), there might be a second decline comparable with the one at low temperature (*S*—*X*).

Possible Interpretations

The results of these experiments show that every factor tested has a very noticeable effect on wing-production. A fair weighting of their effects would undoubtedly assign a greater influence to alternating light and darkness, as against continuous light, during the experiment than to any other agent. Second place would probably be taken by the temperature used (whether 24° or 14°) during the experiment. Third rating would probably go to wings or winglessness in the parents, though its effect is striking only in parents taken from a low temperature stock. And fourth place belongs to the temperature applied to the parents before the beginning of the experiment, the lowest place being assigned to this factor because it acts in a regular and marked way only on the winged parents and only in the two constant temperatures used.

The somewhat rhythmical succession of depressions and peaks of wing-production under the most influential of these agents, namely, alternating light and darkness, indicate that a moderately simple physiological explanation ought to be attainable. Such an explanation should be sought with caution, however. An attempt was made in an earlier study (Shull, 1929) to explain wing-production as due to a substance resulting from the decomposition, in darkness, of another substance produced in the light. The strain then being used for experiments was clone *A* of a later paper (Shull, 1932). As described in the latter paper, clone *A* changed radically in the fall of 1929 to become clone *A'*. It is clone *A'* that furnished the material for the experiments here reported, and wing-production in clone *A'* is in many respects different from that of *A*, even to the extent of directly reversing its response to light. A physiological explanation which fits the results from both *A* and *A'* becomes therefore difficult. It would be possible to postulate curves of physiological change of such shape that their relations to one another could be held to explain most of the facts ascertained in this group of experiments, including the rhythmical change of wing-production in curves *S—X* (perhaps also *G—L*). Until there is some known physiological feature of the aphids which corresponds to at least part of such assumptions, however, the devising of curves is of doubtful value. It seems the part of wisdom to wait.

Summary

In general, continuous light applied to the strain of aphids here used resulted in more wing-production than did alternating light and darkness, mostly regardless of the other conditions imposed.

More winged offspring were produced at high temperature than at low, in all combinations of other conditions except one. The difference in the one exceptional set of conditions was small.

A mere change of temperature from low to high or from high to low may perhaps reduce wing-production in certain of the combinations of other agents, but not in most of them.

Winged parents produced strikingly fewer winged offspring than did wingless parents if taken from the low temperature stock. Winged parents from the high temperature or alternating temperature stocks produced mostly more winged offspring than did wingless parents, but none of the differences was large.

Winged parents taken from a high temperature stock produced many more winged offspring under all other conditions than did winged parents taken from a low temperature stock.

Wingless parents generally reversed the above response, since they produced more winged offspring if taken from a low temperature stock than if taken from a high temperature, in all combinations of other conditions except one. In that one exception there was no difference between the wingless parents from the two different temperatures.

Regarding the response of parents taken from an alternating stock as compared with constant temperature stocks, no general rule can be stated. The results were very irregular.

Under uniform conditions, and without change from the conditions applied to the parents before their reproductive period begins, there is a rather rapid and steady decline in the number of winged offspring from the beginning to the end of the family. The decline is more rapid for wingless parents than for winged ones.

At high or low temperature and in continuous light the age effect described in the preceding paragraph is the chief factor governing distribution of wing-production through the family.

At high temperature and in alternating light and darkness there is a decline in wing-production early in the family, followed by a sharp rise later, regardless of the type of parents or the temperature from which they were taken.

At low temperature and in alternating light and darkness there is a decline of wing-production early in the family, a slight or moderate rise thereafter, and a decline toward the end of the family, regardless of the type of parents or the temperature from which they were taken.

The most effective of all the agents tested in these experiments is the light conditions (whether continuous or alternating) prevailing during the experiment. Temperature during the experiment is next most important. Wings or winglessness of the parents is third in importance,

followed closely by the temperature at which the parents were reared before the experiment began.

The time seems not ripe to attempt a physiological explanation which will fit these results as well as the somewhat divergent ones obtained in previous studies.

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TAXONOMIC AND CYTOLOGICAL STUDIES ON THE CILIATES ASSOCIATED WITH THE AMPHIPOD FAMILY ORCHESTIIDÆ FROM THE WOODS HOLE DISTRICT

I. The Stomatous Holotrichous Ectocommensals

GEORGE W. KIDDER AND FRANCIS M. SUMMERS

(From the Marine Biological Laboratory, Woods Hole, Massachusetts)

This is the first of a series of studies undertaken in an effort to contribute to our knowledge of the ciliates, both commensal and parasitic, that are associated with various invertebrate hosts in the region of Woods Hole, Massachusetts. The present paper deals with the stomatous ectocommensals of three species of the family Orchestiidæ: *Talorchestia longicornis* (Say), *Orchestia agilis* Smith, and *Orchestia palustris* Smith. The study includes not only the morphological details necessary for the determination of the taxonomic rank of this group of ciliates but also a cytological study of the process of binary fission of one of the species.

The material for this investigation was collected in the vicinity of Woods Hole, Massachusetts and the studies carried out at the Marine Biological Laboratory. We wish to express our appreciation to Miss Florence Stuck of Columbia University for calling our attention to the occurrence of some of the ciliates here described.

Material and Methods

Our initial material was collected during the summer of 1931 from Nobska Beach. At this time a number of specimens of the sand flea *Talorchestia longicornis* were taken and transferred in moist sand to New York City where a preliminary study of the ectocommensals was made. During the summer of 1934 intensive collections of the same species of amphipod together with *Orchestia agilis* and *Orchestia palustris* gave us ample opportunity to continue our observations. *Orchestia agilis* and *Orchestia palustris* were also collected from Sippewisset Beach and from the muddy banks of the Eel Pond in Woods Hole.

Talorchestia is found buried in the sands during the day near the high water mark. It is a relatively simple matter to obtain abundant material by digging them out and placing them in a container partially filled with moist sand. *Orchestia agilis* is found in great abundance

among the moist and decaying sea weed that has been beached by the tide. They can be collected by the thousands by shaking some of this sea weed over any vessel, care being taken to cover the vessel quickly to prevent their escape. *Orchestia palustris* burrows in the mud under decaying vegetation and must be collected individually. As this species is much less active than the preceding one, abundant material can be collected in a minimum of time.

In all cases the amphipods were brought directly to the laboratory where the preparations of their ectocommensals were made. As the material was abundant, ample opportunity was afforded for a detailed study in the living condition of all of the species of ciliates here described. For the cytological details permanent preparations were made by crushing the host on an albuminized cover glass, the ciliates coming away from the carapace in the body fluids. These smears were then fixed and stained in any desired manner.

For morphological observation the best results were obtained by fixing the ciliates in strong Flemming's fluid, staining in Heidenhain's hæmatoxylin and differentiating in a 10 per cent solution of superoxol, following the method described by Kidder (1934*a*). Excellent results were also obtained by the use of the Borrel mixture following hot (60° C.) Schaudinn's fluid.

For the details of the divisional activity the Feulgen nucleic-acid reaction following hot (60° C.) Schaudinn's fluid proved invaluable, although each stage was checked with material stained in Heidenhain's hæmatoxylin and differentiated in iron alum.

Taxonomy

Only two genera of holotrichous ciliates are found to be associated as commensals with the three species of amphipod hosts studied. The most abundant ciliates belong to a hitherto undescribed genus, which we have named *Allosphærium* gen. nov., of which there are five distinct species. Less abundant, but occurring regularly, are found three undescribed species of the genus *Chilodonella* Strand.

Allosphærium gen. nov.

Allosphærium is a small (24 μ to 59 μ) holotrichous ciliate. All of the species so far discovered live as commensals on the carapace and gill lamellæ of three species of amphipods (*Talorchestia longicornis, Orchestia agilis,* and *Orchestia palustris*). They are remarkably adapted for this mode of life, being very flat dorso-ventrally and possessing great thigmotactic powers. When washed onto a slide and studied in life it is seen that they adhere tenaciously to the glass or to the surface film of

the water. They creep rapidly along these surfaces with a rather steady motion moving in wide circles to the left. When dislodged they swim rapidly in a jerky fashion and quickly settle to any surface with which they come in contact.

When viewed from the dorsal surface these ciliates are nearly oval, although slightly asymmetrical. The left lateral margin conforms more nearly to the anterio-posterior axis than does the right, which is curved in a wide arc. The anterior end is usually slightly pointed while the posterior end is evenly rounded in most cases, although in some it is nearly truncate.

The dorsal surface is arched and devoid of cilia while the ventral surface is slightly concave and covered with from twelve to twenty-seven rows of fine cilia. On the ventral surface the lateral pellicle is extended into two folds partially enclosing the concave ciliated area. The right fold is smooth and extends nearly or wholly the length of the body, while the left fold is more in the form of a lapel, smooth in some cases, notched in others.

The cytostome is located near the anterior end on the ventral surface of the body. It is oval or irregular in shape depending on the species. The posterior border of the cytostome is guarded by a shelf or ridge, perpendicular to the long axis of the body and extending to the left lateral margin. Anterior to this ridge the ventral surface falls away into a trough which forms a naked, rather shallow oral groove. The cytostome is equipped with peculiar specialized ciliary elements. Originating within the oral opening and extending well to the outside are three groups of fused cilia, forming three separate membranes. These membranes are pointed at their distal ends and fan-shaped proximally. Two of them originate from the posterior wall of the cytostome and somewhat overlap one another while the third is always more narrow and originates from the right wall of the cytostome. The ones posterior beat out and up in a direction parallel to the long axis of the body. The narrow lateral membrane beats out and toward the left in a direction perpendicular to the long axis of the ciliate. The cilia of the membranes are fused and beat synchronously as a single unit but the membranes are not synchronized with each other. Their movements are discontinuous and their function is presumably to sweep food into the mouth in much the same manner as the pseudomembranelles of *Kidderia* Raabe 1934*a* (*Conchophthirius*) *mytili* (Kidder, 1933*a*).

There is a single macronucleus, more or less oval in shape, located near the center or slightly anterior to the center of the cell. The single micronucleus is situated just anterior to the macronucleus.

In four of the five species of this genus there are two well-developed contractile vacuoles, one in the anterior fourth of the body well toward the left margin, and one in the posterior fourth of the body, either centrally located or toward the left margin of the cell. The fifth species possesses but one contractile vacuole which is situated toward the left lateral edge just anterior to the cell center.

In the posterior cytoplasm well toward the left side of the body is found a curious inclusion which is constant for all species of the genus. In life this inclusion is in the form of a refractile sphere while in hæmatoxylin preparations it stains intensely. It decolorizes more readily than do the nuclei but can easily be identified, especially in Flemming-Heidenhain preparations. It is negative to the Feulgen nucleic-acid reaction and does not stain with either neutral red or Janus green. The acid (green) component of the Borrel mixture stains it with great intensity. All fixations used, whether or not they contain acetic acid, preserve it. In life as well as in stained preparations this endoplasmic sphere always appears homogeneous as to structure and constant as to shape. Our observations to date have given us no clue as to the possible significance or function of this unique cell inclusion. Because of its behavior during cell division, as will be noted later in this report, we are inclined to regard it as a metaplastid, but one that is very regular in its appearance within the species of this genus.

The five species of the genus *Allosphærium* form a closely integrated group. They differ from one another in average sizes although there is considerable overlapping at the extremes. The number of rows of cilia on the ventral surface is constant for a given species and this criterion, taken with the differences in shape, size, and appearance of the nuclei as well as the ventral pellicular folds, makes it possible to differentiate one species from another readily.

There appears to be little host specificity in this group as all three species of amphipods used in this investigation were found to carry an infestation of at least two of the species of *Allosphærium*.

Following is a short general description of the five species of the genus *Allosphærium*.

Allosphærium palustris gen. nov., sp. nov. (Fig. 1, *A*).—This ciliate is the largest species of the genus so far encountered. It averages 55 μ in length (46 μ to 59 μ). The dorsal surface is weakly arched and possesses three folds. One fold occurs along the right margin in the posterior half. It follows the general contour of the body and terminates near the posterior end of the ciliate. The other two start just posterior to the level of the cytostome near the left margin and end at about the level of the endoplasmic sphere. These dorsal furrows are constant in

this species. They disappear only after the ciliate becomes flattened, between the slide and cover, to the extent that only feeble motion is possible.

The ventral surface is nearly flat and covered with twenty-seven rows of fine cilia. Of these twenty-seven rows three originate anterior and to the left of the cytostome, curve around its anterior extremity and follow the contour of the right lateral margin of the body and finally end in an oblique suture near the left posterior edge of the ciliate. The remaining twenty-four rows originate in a line just posterior to the cytostome and proceed backwards. Half of them swing down in an arc to enter the suture from its posterior right side, while the other half either converge at the innermost tip of the suture or enter it from its left anterior side. The cilia in the mid-region of the body are rather short while those of the anterior portion about the mouth and those of the extreme posterior region of the cell are quite long. All of these peripheral cilia beat metachronously.

The cytostome is relatively large, the outlines being regularly notched on the right side. The two posterior ciliary membranes are large and triangular while the right membrane is narrow and long.

The right ventral pellicular fold is very narrow and shallow, extending the full length of the body. The left fold starts just anterior to the center of the cell and is extremely broad. It is smoothly rounded and overlaps a considerable portion of the left posterior region of the ventral surface.

The endoplasmic sphere is always situated just at the inner end of the oblique suture. It is relatively small in comparison with the same inclusion of some of the other species of this genus.

There are two contractile vacuoles, one located just anterior and slightly to the left of the endoplasmic sphere and the other situated near the right border of the cell in the anterior fifth of the body. Their diastolic period is quite long and there appears to be no regularity between the emptying of the two.

The macronucleus is ovoid in outline and appears to be made up of a dense reticulum of chromatin. It lies in the anterior portion of the cell slightly to the right of the center. The single micronucleus is found just anterior to the macronucleus. It is relatively large and spherical and stains very intensely with all of the basic dyes.

Allosphærium palustris is found commonly although never abundantly on the carapace and gill lamellæ of *Orchestia palustris*. In a small number of specimens of *Talorchestia longicornis* a few ciliates of this species were encountered. We have never obtained this species of ciliate from *Orchestia agilis*.

Allosphærium sulcatum gen. nov., sp. nov. (Fig. 1, *B*).—This species is the smallest member of the genus. In length it averages 26 μ, the range being between 24 μ and 32 μ. The most characteristic feature to be noted is the presence of a deep groove or sulcus on the dorsal surface well toward the left margin. This sulcus extends from the level of the cytostome nearly to the posterior end of the organism. It makes a sharp dip to the left near its distal end.

The ventral ciliated surface is somewhat restricted in this species due to the development of the lateral pellicular folds. The right fold encloses approximately one-fifth of the ventral surface while the lapel-like left fold extends over a portion of the endoplasmic sphere. The pellicular folds are continuous around the posterior end in *A. sulcatum*, producing a deep rounded notch at the posterior end of the lapel.

There are only twelve rows of cilia in this species. They are arranged in the same general manner as those of the preceding species. We have never been able to detect the presence of a posterior suture, however, but this may be due to the obscuring effect of the pellicular lapel.

The cytostome is similar in shape and position to that of *A. palustris*. The ciliary membranes are arranged in the same order but the two posterior ones are more attenuated at their proximal ends.

The nuclei and the contractile vacuoles of *A. sulcatum* are similar in relative size and position to those of *A. palustris*.

Allosphærium sulcatum is found regularly but in small numbers on the carapace of *Orchestia agilis* and *Orchestia palustris*.

Allosphærium granulosum gen. nov., sp. nov. (Fig. 1, *C*).—This species is characteristically more rotund than any of the other members of the genus. When viewed from the ventral side its shape is nearly oval and the width of the body is approximately three-fifths that of the length. The smooth dorsal surface is highly vaulted, making this organism quite thick dorso-ventrally. The ciliated portion of the ventral surface is nearly flat. The position and extent of the ventral pellicular folds are like those of *A. sulcatum*, although the left lapel does not extend quite to the endoplasmic sphere.

The ventral surface possesses seventeen rows of cilia originating as

FIG. 1. All figures are composite drawings from living and stained material, drawn from the ventral side. × 1500.

A. Allosphærium palustris gen. nov., sp. nov.
B. Allosphærium sulcatum gen. nov., sp. nov.
C. Allosphærium granulosum gen. nov., sp. nov.
D. Allosphærium caudatum gen. nov., sp. nov.
E. Allosphærium convexa gen. nov., sp. nov.
F. Chillodonella hyalina sp. nov.
G. Chilodonella rotunda sp. nov.
H. Chilodonella longipharynx sp. nov.

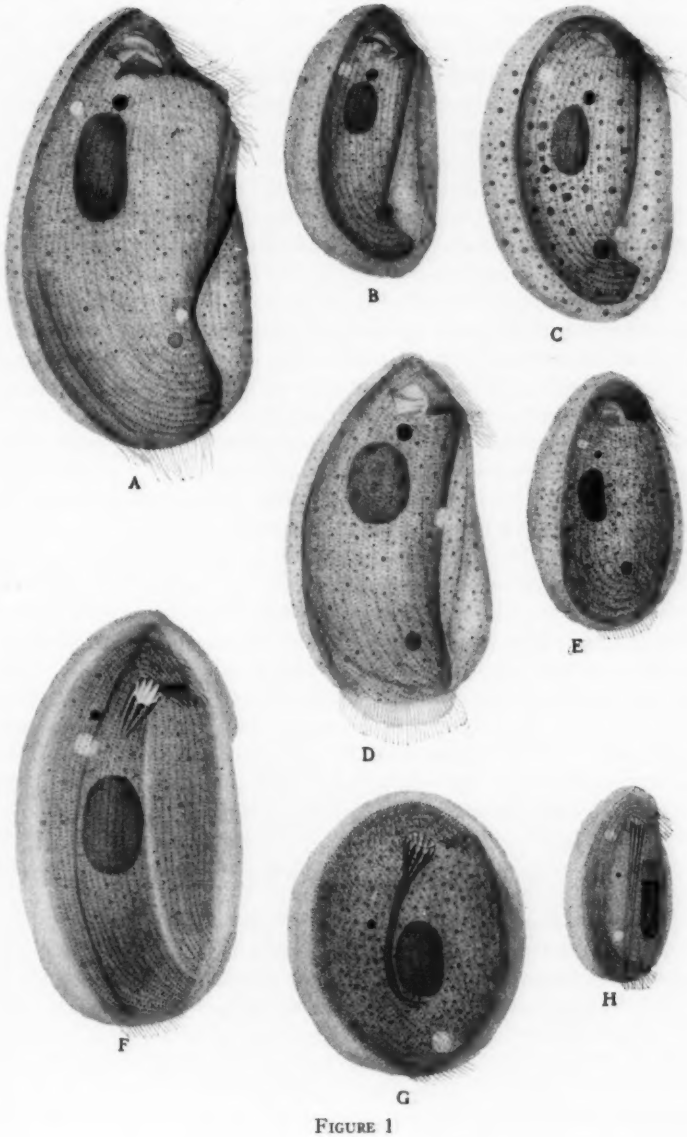

FIGURE 1

in the preceding species. Here again we were unable to detect the presence of a posterior suture probably because of the overfolding lapel.

The cytoplasm of *A. granulosum* is characteristically filled with large granules that render this organism semi-opaque. These granules together with the thickness of the body enable the observer to identify this species with ease.

The nuclei, contractile vacuoles, and cytostomal structure are practically identical with those of *A. sulcatum*.

Allosphærium granulosum averages 38 μ in length, the extremes being between 32 μ and 42 μ. It is found regularly in small numbers on the carapace of *Orchestia agilis* and *Orchestia palustris*.

Allosphærium caudatum gen. nov., sp. nov. (Fig. 1, *D*).—This species resembles *A. palustris* in general body outline. The ventral surface is slightly concave and possesses fourteen rows of cilia. The dorsal surface is weakly arched. A sulcus runs obliquely from near the center of the right margin to the left posterior edge of the organism. Another shallow furrow starts at the level of the cytostome on the left cell border and continues posteriorly nearly the length of the body. It bends somewhat to the left near its posterior extremity. The bend is exactly the reverse of that noted in the case of *A. sulcatum*.

The pellicular fold of the right ventral margin is narrow, as in *A. palustris*, and is of similar extent. The left fold is quite wide, starting at the level of the cytostome and extending to the posterior end. There is no notched appearance in the left fold, the border of which curves slightly to the right at its posterior extremity.

The nuclei resemble those of *A. palustris* except that the micronucleus is somewhat larger and the macronucleus is more spherical than ovoid. There is but one contractile vacuole located well toward the left border of the cell and slightly anterior to the center. This is the only case so far encountered in this genus where there is a single contractile vacuole.

The cytostome and its ciliary apparatus are almost identical with those of *A. sulcatum* and *A. granulosum*.

The most characteristic feature of this species and one that enables the observer to recognize it with ease is the condition of the posterior ectoplasm. Across the posterior end the ectoplasm is drawn out into a shelf. This caudal shelf is very transparent and does not stain with any of the dyes. The posterior cilia, however, are very long and extend under the shelf so that in stained preparations (Heidenhain's hæmatoxylin) the shelf resembles a little fan.

Allosphærium caudatum is found sparsely on the carapace and gill lamellæ of *Orchestia agilis*. We have never encountered it on either

of the other two amphipods used for this study. It averages 41 μ in length, the extremes being 35 μ to 45 μ.

Allosphærium convexa gen. nov., sp. nov. (Fig. 1, *E*).—Because of its regular occurrence and abundance on *Talorchestia longicornis* we have designated this as the type species of the genus in spite of its small size.

As viewed from the ventral side this species is nearly egg-shaped, the narrower end being the anterior. The ventral surface is weakly concave and is supplied with seventeen rows of cilia, arranged in a manner similar to those of the preceding species. Both the right and particularly the left ventral pellicular folds are so narrow that practically the whole ventral surface is exposed. Here the posterior suture is clearly visible. It is an oblique line running anteriorly and to the right from the small notch at the junction of the right and left folds to the endoplasmic sphere.

The cytostome of *A. convexa* is proportionately smaller than any of the other species and is in the form of a nearly circular opening. It is supplied with the usual three ciliary membranes closely resembling those of *A. palustris* as to shape and distribution.

The macronucleus is bean-shaped and lies somewhat to the right near the middle of the body. It is smaller than the macronuclei of any of the preceding species. The micronucleus is minute and occupies a position just anterior to the macronucleus.

There are two contractile vacuoles, the anterior one in the usual position toward the right margin of the cell but the posterior one is found at about the mid-line of the body to the right of the endoplasmic sphere.

Allosphærium convexa averages 29 μ in length. The extremes encountered were from 24 μ to 36 μ. It is found in great abundance on the carapace and gill lamellæ of *Talorchestia longicornis*. We have never encountered it on either *Orchestia agilis* or *Orchestia palustris*.

Chilodonella Strand (Chilodon Ehr.)

Associated with the various species of the genus *Allosphærium* on the carapace of the three amphipods used in this investigation are to be found three species of the genus *Chilodonella*. A large percentage of the hosts examined were found to harbor a few of these ciliates. They are all sufficiently different from previously described species of the genus, as far as we have been able to determine from the literature, to warrant short descriptions. For some time we were in doubt as to the commensal nature of these ciliates, thinking they might represent vagrant free-living forms. A search of the sand and sea weed failed

to disclose any ciliates of like nature, however, and this fact together with the general similarities of the *Chilodonella* and the *Allosphærium* convinced us that we were dealing with forms that normally lived as commensals on the carapace of the amphipod hosts. They live but a short time in sea water when washed free from their host, a characteristic of all species of the genus *Allosphærium*.

Chilodonella hyalina sp. nov. (Fig. 1, *F*).—This species is flattened ventrally and convex dorsally. The dorsal surface bears a longitudinal ridge that extends from near the anterior end and curves toward the right to the posterior end. The lateral margins of the ciliate are extended into a hyaline shelf which reminds one of the brim of a hat. This shelf extends completely around the body except for a short space at the extreme posterior end. It is quite thin and seems to be entirely without visible structure.

The cilia are confined to the ventral surface, as in all members of this genus. They are arranged in twenty-three rows. Nine of these rows originate from the anterior side of a line that extends from the mouth to the left margin of the ciliated part of the ventral surface. These nine rows pass anteriorly and to the right, form an arc above the mouth and then pass down the right margin of the cell to end well over toward the left posterior edge. On the left side of the ciliated area eight rows of cilia originate from the posterior side of the short transverse line. They pass backward and bend slightly to the left. The remaining six rows appear to originate from the region of the mouth. There is a sharp line of demarcation between the eight rows of the left side and the rest of the rows of the ventral surface. These two areas are separated by a naked band that extends from the mouth to the posterior end. In life this band is clearly visible as a light streak separating the ciliated area into two parts. Along the line that extends from the mouth transversely to the left margin arise a row of large, stiff cilia. These cilia beat much more leisurely than do the rest of the body cilia.

The mouth is surrounded by a ring of trichites forming a complete pharyngeal basket. The basket is quite short in this species and extends obliquely into the endoplasm. The trichites taper toward their inner ends and disappear from view among the endoplasmic granules. As in the free-living *Chilodonella*, the pharyngeal basket stains deeply with hæmatoxylin. It is also very conspicuous after the Borrel stain where it takes the acid component (green).

The macronucleus is centrally located. It is oval and is relatively large. The chromatin is evenly dispersed in fine granules without any trace of an "endosome" (see MacDougall, 1925, for a description of

this body in *Chilodon uncinatus*). The micronucleus is located just to the right of the pharyngeal basket.

There are two contractile vacuoles, one just below the micronucleus and the other near the posterior end to the left of the naked band.

Chilodonella hyalina averages 40 μ in length. The extremes are between 36 μ and 47 μ. It is found exclusively on the carapace of *Orchestia agilis*.

In life *Chilodonella hyalina* resembles to a marked degree the various species of the genus *Allosphærium* and without the aid of the oil immersion lens to note the pharyngeal basket it might well be mistaken for a member of that group.

Chilodonella rotunda sp. nov. (Fig. 1, *G*).—The ventral surface of this species is flat but it is strongly arched dorsally. In side view the organism resembles a derby hat with a very narrow brim, while from the ventral surface it appears nearly round. The brim is not hyaline, as it is in the preceding species, but is somewhat granular.

The cilia are arranged much in the same manner as those of *C. hyalina* but the rows are not separated into two groups. There are twenty rows, five of which originate from the anterior side of the transverse oral line.

The pharyngeal basket is quite different from that of *C. hyalina*. It is composed of trichites that show distinct thickenings near their anterior ends. The basket itself is flared out around the mouth. It narrows rapidly into a long gullet supported by the distal ends of the trichites. The gullet extends into the posterior fourth of the body, curving sharply to the left.

The macronucleus is ovoid and is densely and homogeneously granular. It always lies a little back of the center within the curve of the gullet. The micronucleus lies near the center of the cell to the right of the gullet.

There is a single large contractile vacuole very near the midline at the posterior end of the body. The diastolic period is quite long.

Chilodonella rotunda averages 29 μ in length. The extremes were found to be from 27 μ to 34 μ. It is nearly as wide as it is long. So far we have found it only on the carapace of *Orchestia agilis* and never more than two or three specimens on each host infected. It is easily recognized by its general shape, the shape of the pharyngeal basket, and the dark coloration due to the thickness of the granular endoplasm.

Chilodonella longipharynx sp. nov. (Fig. 1, *H*).—This is the smallest representative of the holotrichous ectocommensals found on this group of amphipods. Its reduced size together with its almost crystalline clearness makes it an exceedingly difficult organism to study in life. In

material stained in Heidenhain's hæmatoxylin or the Borrel stain, however, the details of its structure can easily be observed.

The ventral surface is naked except for four rows of rather long cilia. These rows originate anterior to the transverse oral line and describe a semi-circle, up and along the left margin, across the anterior end, down the right margin and thence left across the posterior end. The cilia that originate near the transverse oral line are quite long and relatively thick. As in *C. hyalina* they beat more slowly than do the rest of the cilia along the four rows.

The mouth is surrounded by an extremely long pharyngeal basket which extends nearly to the posterior end of the body. It is made up of straight trichites, which render the basket cone-shaped. In hæmatoxylin preparations the pharyngeal basket is the most conspicuous structure in the cell.

There are two contractile vacuoles both lying to the right of the pharyngeal basket. One is just below the level of the cytostome while the other is in the posterior fifth of the body.

The macronucleus is an elongate oval and is situated along the left margin of the cell. In the majority of ciliates of this species examined there appears a band, very similar to the Kernspalt of numerous hypotrichous forms, extending across the macronucleus. The chromatin on either side of the band is of distinctly different structure, the anterior chromatin being finely granular and faintly staining while the posterior chromatin is made up of a coarse reticulum and stains deeply. The band itself is made up of two parts, a clear plane and an intensely staining plane. This structure is obviously bound up with some stage of development of the ciliate, probably with binary fission. We have found stages where the band was very near to the anterior end of the nucleus and in all gradations of position to about the posterior sixth. Whether the band passes off the end of the nucleus, as it does in a number of hypotrichous forms (see Summers, 1935), or not we cannot say. Although we have hundreds of organisms of this species stained, we have not observed the actual fate of the band. As the band reaches about the halfway point in the nucleus, a deeply-staining sphere is differentiated in the finely granular (reorganized?) portion. This sphere increases in size until its diameter reaches about one-fourth the width of the macronucleus. We cannot say what is the significance or fate of this sphere. We are aware of no other species of *Chilodonella* that shows this band, although it is identical with the one found in *Trochilia* (*Dysteriopsis*) *minuta* described by Roux (1901) and Penard (1922) and seems to be similar to those of a number of species of *Chlamydodon* described by Kahl (1931).

The single micronucleus is very small and is always situated in about the center of the cell on the opposite side of the pharyngeal basket from the macronucleus.

Chilodonella longipharynx averages 19 μ in length, the extremes being from 17 μ to 21 μ. It is found most abundantly on the carapace of *Talorchestia longicornis* and less frequently on *Orchestia palustris*. We have never encountered it on *Orchestia agilis*.

Discussion of Taxonomic Affinities

The genus *Allosphærium* obviously belongs to the sub-class Holotricha Stein (Calkins, 1933) and by virtue of the membranes in the mouth must be included in the order Hymenostomida Hickson (Calkins, 1933). But the allocation to any of the established families of that order offers grave difficulties. In none of the members of the order so far described does there occur a restriction of the cilia to the ventral surface. It seems inescapable, therefore, that a new family should be erected for the reception of the genus *Allosphærium*. We are loath to do this at this time, however, because we feel that the affinities for at least one other form that has previously been described are too close to be disregarded, and as yet the existing description is too fragmentary to warrant an analytical comparison. The form in question is *Lophophorina* Penard 1922. This genus, erected for the reception of a single species (*L. capronata*), resembles *Allosphærium* in size, shape, distribubution of cilia, movements and location on its host (fresh-water *Gammarus*). Indeed we were at first of the opinion that we were dealing with species of the same genus as described by Penard. But when it was seen that our forms all conformed so closely to a set pattern which was different in many important respects from *Lophophorina* we were forced to conclude that we were dealing with a new genus. Penard (1922, p. 96) was unable to locate the mouth but he assumed it to be in the anterior portion of the cell near the long "tentacle." By this it can be readily seen that no diagnosis as to its order is possible without knowledge of so important a structure as the cytostome. Still we feel that there is a possibility that when *Lophophorina* is re-investigated it may be possible to erect a family for the reception of both genera (*Lophophorina* and *Allosphærium*), in which case the family name should be, because of priority, Lophophorinidæ. For the present, therefore, we wish to defer the action of establishing a new family.

The previously described species of the genus *Chilodonella* that resemble most closely those found by us on the amphipods studied are those of Penard (1922). His *Chilodonella* (*Chilodon*) *capucinus* (p. 92) possesses two contractile vacuoles as do our *C. hyalina* and *C.*

longipharynx. It has a spherical macronucleus and the cilia are divided into two areas. There are only ten ciliary rows, however, (five right and five left) as contrasted with the twenty-three rows of *C. hyalina*. The shape of the macronucleus and the ciliation set it apart from *C. longipharynx* while the possession of ten ciliary rows as opposed to twenty for *C. rotunda* together with the difference in contractile vacuole number show it to be distinct from the latter species. *Chilodonella* (*Chilodon*) *granulatus* (Penard, 1922, p. 95) has the same type of ciliation as *C. longipharynx* but there are from five to seven rows. The pharyngeal basket is short and re-curved and the cell possesses but one contractile vacuole. Both *C. capucinus* and *C. granulatus* are ectocommensal on *Asellus* and *Gammarus*.

It should be pointed out that the general shape of the holotrichous ectocommensals of both the amphipods and isopods that have so far been described is singularly well adapted for their environment. They are all small flat forms and possess ventrally placed thigmotactic cilia (*Chilodonella, Trochilia, Allosphærium*). When one considers the forces, mainly in the form of water currents, to which they must be subjected and which would tend to effect their removal from the carapace of their various hosts, it is seen that the flatness of their bodies and the adhesive powers of their ventral cilia are of absolute necessity. Existing under the same conditions, it is perhaps not surprising that representatives of two orders of ciliates exhibit convergence to such a degree as to render them practically indistinguishable one from the other except under extreme magnifications.

Division of Allosphærium convexa gen. nov., sp. nov.

Although we have encountered cases of binary fission in practically all of the species described above, we have been able to trace the process completely in only one form, *Allosphærium convexa*. This species occurs in relatively large numbers and we have had an opportunity to study many hundreds of specimens. Of these a rather high percentage were in some phase of binary fission.

Our observations on the process of division of this minute ciliate are confined mainly to the behavior of the macronuclear chromatin. The cytoplasmic structures and the micronucleus are too small to permit us to follow all of the details of their divisional activity.

The chromatin of the macronucleus appears as a dense reticulum during the resting period (Fig. 2, *A*). It stains intensely with all of the nuclear dyes. With the Feulgen nuclei-acid reaction the reticulum appears to enclose many vacuole-like spaces of varying sizes.

The first sign of fission is to be seen in the peculiar activity of the

central portion of the macronuclear chromatin. A core of chromatin forms in the center of the nucleus, becomes very finely granular, and loses, to some extent, its affinity for basic dyes. A clear zone surrounds

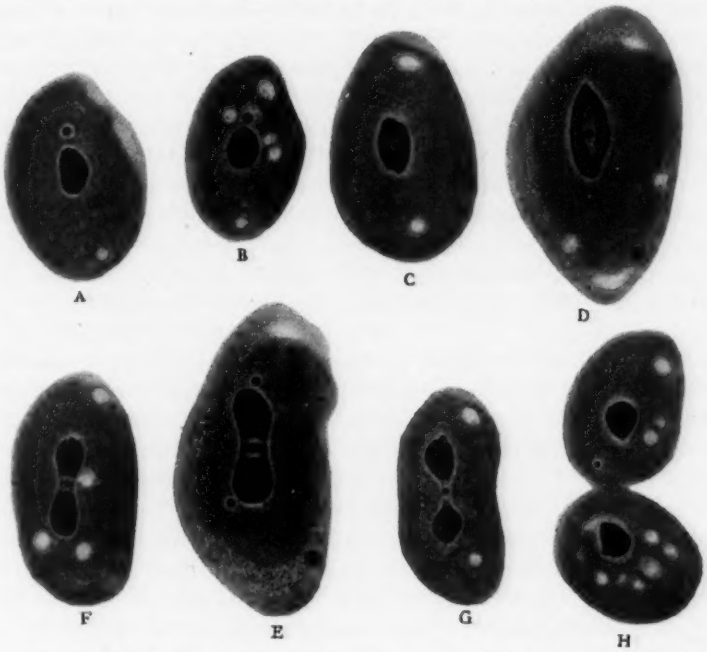

FIG. 2. All figures are of *Allosphærium convexa* during binary fission. The figures represent optical sections of stained organisms drawn with the aid of the camera lucida. × 990.

A. Resting stage. The macronuclear chromatin in the form of a coarse reticulum. Schaudinn-Feulgen.

B. Formation of the central chromatin ball. The shell of reticular chromatin is shown only at the edges in this optical section. Flemming-Heidenhain.

C. Contraction of central ball. Note the concentration of reticular chromatin at poles. Schaudinn-Feulgen.

D. Elongation of macronucleus. Daughter micronuclei below the level of focus. Flemming-Heidenhain.

E. Constriction stage. Most of the reticular chromatin in bands near the division plane. Flemming-Heidenhain.

F. Later stage. The central ball has become loosened and spread out between the future daughter macronuclei. Schaudinn-Feulgen.

G. Separation stage. Schaudinn-Feulgen.

H. Macronuclear division completed. The central ball is cast into the cytoplasm, in this case, of the anterior daughter. Schaudinn-Feulgen.

it, separating it from the shell of unchanged reticular chromatin (Fig. 2, *B*). At this time the activity of the micronucleus is evidenced by an increase in size and by dispersal of its chromatin as fine granules.

The central core of the macronucleus gradually contracts and becomes basophilic, ultimately forming a small sphere in the center of a relatively clear region. In the meantime the reticulate shell clumps into large chromatin masses, with the major portion passing to each pole as the nucleus elongates (Fig. 2, *C*). As elongation proceeds the mid-region, with the exception of the central sphere, becomes clear and nearly free from chromatin. Slightly later the chromatin of the reticulum is found massed at the two poles, connected, however, by numerous fine strands that pass around the clear mid-region (Fig. 2, *D*).

As the macronucleus begins to constrict, the entire chromatin content behaves in an unusual manner. Most of the "active" chromatin forms long, thread-like bodies and migrates toward the division plane, leaving, however, two small polar caps. On either side of the division plane the thread-like bodies form two plates, resembling chromosomes in the anaphase (Figs. 2, *E* and *F*). Their appearance is never regular, however, and we would certainly hesitate to call these bodies macronuclear chromosomes. The chromatin of the central ball loses its compactness and spreads out across the division plane. It is always quite definite and stains clearly after the Feulgen reaction as well as with the basic dyes (Fig. 2, *F*). Further constriction results in a second contraction of this "waste" chromatin to form the residual ball. The chromosome-like bodies of the daughter macronuclei become very irregular and more or less fusion of these bodies with the chromatin of the polar caps ensues (Fig. 2, *G*). As the daughter macronuclei separate, the residual ball of chromatin is cast into the cytoplasm (Fig. 2, *H*) where it decreases in size and is finally absorbed. The chromatin of the daughter macronuclei gradually assumes the structure of the resting reticulum as reorganization proceeds. Plasmotomy proceeds to completion and the daughter ciliates separate.

It may be well to mention the appearance of the endoplasmic sphere during cell fission. There is apparently no division of the sphere since it may be identified in its original position during all division phases. It does not change appreciably in size or staining capacity. The new endoplasmic sphere of the anterior daughter seems to arise *de novo* just anterior to the plane of fission. It may be seen first as a faintly staining area (after Heidenhain's hæmatoxylin) which gradually contracts into a sphere. This would lead one to believe that the endoplasmic sphere is a metaplastid representing some product of metabolism. At least it does not appear to be a self-perpetuating structure.

Discussion of Division

In the light of a number of recent reports the extrusion or elimination of chromatin or chromatin-like materials from the macronucleus during binary fission is not particularly unusual. This phenomenon occurs in a number of holotrichous ciliates, e.g., *Loxocephalus* (Behrend, 1916); *Eupoterion* (MacLennan and Connell, 1931); *Kidderia* (*Conchophthirius*) (Kidder, 1933*a*); *Ancistruma* (Kidder, 1933*b*); *Ichthyophthirius* (Haas, 1933); *Conchophthirius* (Kidder, 1934*b*); *Urocentrum*, *Colpidium*, and *Glaucoma* (Kidder and Diller, 1934). One case that shows striking similarities to that of *Allosphærium convexa* is that described by Rossolimo and Jakimowitsch (1929) in the division of *Myxophyllum* (Raabe, 1934*b*). This ciliate was known until recently as *Conchophthirius steenstrupii* and possesses seven macronuclei in the normal vegetative stage. During division each of the macronuclei, in addition to casting out a part of the chromatin substance, is described as undergoing a type of mitosis. Most of the chromatin takes the form of two groups similar to the chromosomes of a metazoan during the anaphase. There are also two polar caps of chromatin. We have found a number of instances in *Allosphærium convexa* which nearly duplicate the condition in *Myxophyllum*. With such figures at hand one is tempted to draw analogies with true mitosis.

Summary

1. Three species of the amphipod family Orchestiidæ were investigated for ectocommensals. These forms are *Talorchestia longicornis* (Say), *Orchestia agilis* Smith, and *Orchestia palustris* Smith. The material was collected in the vicinity of Woods Hole, Massachusetts.

2. The stomatous, holotrichous ciliates that live as ectocommensals on the amphipods studied belong to two genera: *Allosphærium* gen nov., of which there are five species, and *Childonella* Strand, of which there are three new species.

3. All species of ciliates are well adapted to their environment, being small and flat and possessing ventrally placed thigmotactic cilia.

4. The binary fission of one species (*Allosphærium convexa* gen. nov., sp. nov.) is described. A ball of chomatin is differentiated in the macronucleus and extruded into the cytoplasm just prior to cell fission. Most of the macronuclear chromatin takes the form of long chromosome-like bodies during division, duplicating to a fair degree the condition found in *Myxophyllum*.

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HYDROGEN-ION CONCENTRATION AND THE RHYTHMIC ACTIVITY OF THE NERVE CELLS IN THE GANGLION OF THE LIMULUS HEART

IPING CHAO

(From the Marine Biological Laboratory, Woods Hole, and the Department of Physiology, University of Chicago)

The influence of the hydrogen-ion concentration on the rhythmic activity of the nerve cells in the respiratory center of the vertebrates has been much discussed (Gesell, 1925). Another form of rhythmic activity of nerve cells is represented by the regular nervous impulse originating in the ganglion of the *Limulus* heart, and the influence of hydrogen-ion concentration on these nerve cells can be studied with great convenience. In 1906 Carlson made a preliminary study of the action of acids and alkalies on the ganglionic rhythm. He observed that addition of 1 part of a 0.6 N NaOH or KOH to 100 parts of sea water or plasma had a stimulating effect on the rate and strength of the nervous discharge from the ganglion. A main factor in this effect, however, appears to be the removal of the Mg-ion as $Mg(OH)_2$, the precipitation of which is readily seen in such an alkaline medium. Carlson also pointed out the difference between the action of strong bases like KOH and NaOH and weak bases like NH_4OH. No such difference, however, was observed with strong and weak acids, including HCl, H_2SO_4, oxalic, citric, acetic, tartaric, malic and formic acids. All these acids produce a primary increase in amplitude and frequency of the heart beat, followed by cessation of the rhythm (Carlson, 1906). Carbonic acid apparently acts in a different way, for Newman (1906) observed a primary increase in the amplitude of the heart beat, associated with a marked decrease in frequency, when CO_2 was bubbled through the sea water surrounding the ganglion. More recently, Asher and Garrey (1930) have repeated Newman's work; they found that CO_2 often had little effect on the ganglion for some time, then quite suddenly the contractions became seriously impaired. No particular attention was paid to the influence of hydrogen-ion concentration as such in these earlier experiments. Variation of either the undissociated acid or the hydrogen-ion concentration in the medium (or of both) might conceivably be factors in the quantitative differences just referred to. Accordingly it seemed desirable to repeat some of these observations and to study further the relation of the hydrogen-ion concentration

to the rhythmic activity of the ganglion in solutions of strong and weak acids and bases.

The experiments to be described were all carried out on the ganglion of the *Limulus* heart. The ganglion was isolated posteriorly, while remaining in connection anteriorly with two segments of the heart musculature. The muscle was kept immersed in sea water and the contractions were recorded graphically. Test solutions were applied to the ganglion alone, and the hydrogen-ion concentrations were determined colorimetrically.

In the study of the influence of the hydrogen-ion concentration, two kinds of effects should be distinguished: (1) the action of the hydrogen-ion in the medium as such, and (2) the secondary effect of other ions or molecules introduced with the acid or base. For the simple effect of the hydrogen-ion concentration as such, both strong mineral acid and base (e.g., HCl and NaOH) and buffer solutions were used. The effects of weak acid and base were also studied to bring out the difference between the penetrating and the non-penetrating substances.

The Action of Acids

The automatic rhythm of the ganglion is not very responsive to changes of hydrogen-ion concentration as such. Addition of HCl to the unbuffered Ringer's solution[1] (Chao, 1933) produces no well-marked change on the ganglion until the acid reaches a more or less toxic concentration of about N/1,000 HCl. At this concentration the rate of the nervous discharge is accelerated considerably; the intensity of the discharge (as measured by amplitude of contraction) may be slightly increased at first but rapidly diminishes; and the rhythm becomes irregular, showing definite symptoms of injury. Experiments with Ringer's solution buffered with primary and secondary Na-phosphate (3 millimols PO_4 per liter) at pH 5.4 also showed no marked effect.

When CO_2 is bubbled through the Ringer's solution or sea water, the reaction of the solution becomes distinctly acid, but the effect on the ganglion is entirely different from that produced by HCl. As described by Newman (1906), the amplitude of the heart-beat may slightly increase at first; later it decreases with decreasing frequency of the nervous discharge. The degree of these changes appears to depend more upon the concentration of the undissociated carbonic acid molecules than upon the acid reaction. Thus, the average rate of the nervous discharge in 10 minutes for two experiments is −14 per cent

[1] The unbuffered Ringer's solution, containing 445 millimols NaCl, 8.9 millimols KCl, and 37 millimols $CaCl_2$ in a liter, is slightly alkaline in reaction like the sea water (pH about 8.2), due to traces of $Ca(OH)_2$ in the $CaCl_2$.

at pH 6.2 and – 38 per cent at pH 5.7 as compared with the normal rate in sea water (pH 8.2). At pH 5.2 (at which sea water is practically saturated with CO_2) the rhythmic activity is inhibited in about 5 minutes (average of 7 experiments). The same pH (5.2) can be obtained by addition of about 2 millimols HCl to a liter of sea water, but this solution produces a slight increase in rate; the average rate in 10 minutes for two experiments is increased by 10 per cent as compared with the normal rate in sea water.[2] When the pH is decreased to 4.0 by addition of more HCl, the average rate in 10 minutes is increased by 17 per cent (average of 4 experiments). Further addition of HCl (3 millimols per liter sea water) may increase the rate 50 per cent or more, often followed by serious impairment of the rhythmicity. These experiments indicate clearly that the external hydrogen-ion concentration is not the determining factor in the action of the CO_2-saturated solution. The action of this medium must apparently be attributed to the undissociated carbonic acid molecules which are known to penetrate living cells with great readiness.

Acetate buffer (10 millimols acetate per liter) dissolved in sea water or Ringer's solution produces the same general effect but appears to be less effective than the CO_2-saturated solutions at the same pH. Thus at pH 5.2, the CO_2-saturated sea water inhibits the rhythm in about 5 minutes on the average, but the acetate-buffered sea water produces only a decrease of 24 per cent of the average rate in 10 minutes (average of two experiments).[3] The concentrations of acetic acid and carbonic acid in the acetate-buffered and CO_2-saturated sea water respectively are, however, not the same; that of carbonic acid being much the higher. The difference in the concentration of the undissociated acid molecules may account for this quantitative difference in action. Further experiments were performed in which different dilutions of acetate buffer were used at approximately the same pH; these showed clearly that the effect on the rhythm increases with increase in the concentration of acetate buffer.

The Action of Alkalies

Experiments with alkali in sea water can be performed within a rather narrow range of pH only, for Mg, which is present in high concentration (up to 53 millimols per liter) is precipitated at $Mg(OH)_2$ near pH 10. Many calcium salts (e.g., carbonate and phosphate) are also very slightly soluble in alkaline solution. Most of the experiments

[2] Lactic acid acts like HCl in causing a primary increase in rate followed by decline and irregularity of beat, but is less effective.

[3] Acetate-buffered sea water can also inhibit the ganglionic rhythm but at a lower pH; the rhythm is inhibited in about 3 minutes at pH 4.4 (average of 5 experiments).

on the action of alkalies were performed by adding the alkali to the simple Ringer's solution which does not contain any Mg-ion.

The pure effect of OH-ions as such can be demonstrated by adding NaOH to the Ringer's solution. No well-marked effect is seen until the concentration of NaOH reaches N/1,000 or more.[4] In a Ringer's solution containing N/1,000 NaOH, there is either no change in frequency and amplitude or a slight decrease in frequency with a slight increase in amplitude. As alkalinity is still further increased, the decrease in frequency becomes more pronounced, the rhythm becomes irregular, and definite symptoms of injury appear.

Similar experiments were performed with NH_4OH to furnish a comparison between strong and weak alkalies. As described by Carlson (1906), addition of 1 part of a 0.6 N NaOH solution to 4,000 parts of sea water or plasma produces a rapid decrease in the intensity of the nervous discharge with a slight acceleration of rate. The same effect is obtained with NH_4OH in the Ringer's solution; with a somewhat higher concentration of NH_4OH (1 millimol per liter) the rhythmic activity is rapidly inhibited, often to complete cessation of rhythm. This inhibition is temporary. If the ganglion is allowed to remain in the solution containing NH_4OH, a gradual recovery follows; both the rate and the amplitude begin to increase and may finally attain a level well above the initial condition. The temporary inhibitory action of NH_4OH resembles the paradox phenomenon shown under certain conditions in the ganglion (Chao, 1934). The effect, however, is not due to the OH-ion, for it cannot be produced simply by adding sufficient NaOH to inhibit the rhythm completely. Nor can it be referred to the action of the NH_4-ion, for NH_4Cl has an entirely different effect on the ganglion. The addition of 5 millimols NH_4Cl in a liter of Ringer's solution or sea water causes a definite increase in the frequency of the nervous discharge[5] (*cf.* also Carlson, 1906). Apparently the peculiar behavior of NH_4OH is to be attributed to the specific action of the undissociated molecules of NH_4OH. These penetrate living cells readily, in contrast to the lack of penetration or slow penetration of strong alkalies in dilute solution.

In general, the results described in these experiments bring out

[4] Ringer's solution buffered with boric acid and NaOH (2.5 millimols borate per liter) at pH 9.4 has no marked effect on the ganglion.

[5] NH_4Cl is slightly hydrolyzed so as to make the solution more acid. However, the slight stimulating action of NH_4Cl is not an effect of the acid reaction, for the solution can be kept alkaline by addition of a small amount of NH_4OH and yet produces the same effect on the ganglion; e.g., the addition of 0.2 millimol NH_4OH together with 5 millimols NH_4Cl to a liter of Ringer's solution (pH 9.2) still increases the rate. The action of NH_4-ion is, in fact, more like that of K-ion (Chao, 1933).

clearly the difference between the respective influences of weak and strong acids and alkalies on the ganglionic rhythm. The characteristic effects of HCl and NaOH on the rhythm are to be referred to simple changes of pH in the external medium; while in the case of weak acids (like acetic and carbonic acids) or weak bases (like NH_4OH) not only are their physiological effects different from those of strong acid and strong base, but their mode of action indicates the presence of special factors, connected apparently with the penetration of the undissociated molecules into the cell interior.[6]

Summary

The automatic rhythmic activity of the nerve cells in the ganglion of the *Limulus* heart is relatively resistant to changes in pH in the external medium as such. Experiments with strong acid and base indicate that no well-marked physiological effect is seen until toxic concentrations are approached. Thus H-ion (N/1,000 HCl) produces a rapid irregular rhythm with decreasing amplitude of contraction followed by cessation of heart-beat, while OH-ion (N/1,000 NaOH) has an inhibitory effect. Weak penetrating acids (e.g., carbonic and acetic acids) inhibit the rhythm with primary increase in intensity of the nervous discharge, while the action of NH_4OH is characterized by a period of temporary inhibition. The contrast is to be related to the characteristic difference in the readiness of penetration of the undissociated molecules and the ions into living cells.

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PITUITARY-INDUCED SEXUAL REACTIONS IN THE ANURA

ROBERTS RUGH

(From the Marine Biological Laboratory, Woods Hole, Mass.)

Since the original work by Wolf (1929), ovulation has been successfully induced by anterior pituitary treatment in sixteen species of Anura and four species of the Urodela (Rugh, 1935). It is the purpose of this paper to present data on anterior pituitary-induced sexual reactions during the summer months. As a result of these observations it is now possible to say that anuran eggs and tadpoles are available at all seasons.

Material and Method

The material used was the five species of Anura available in the Woods Hole region and quite generally in the eastern part of the United States. The forms were:

1. *Rana clamitans* (Latreille), the green frog. The normal breeding season starts about the first of June and continues to about the middle of August. Forms caught during June and July which still have their eggs may be kept in the cold room for several weeks before inducing ovulation or amplexus. Very few of the green frogs caught after the first of August will still have their eggs. These eggs cannot readily be distinguished from those of *Rana pipiens*. A single female will give more than 2,000 eggs during a single season.

2. *Rana catesbiana* (Shaw), the bullfrog. Females of this species with body length up to 120 mm. may show rudimentary ovaries but the immature eggs lack pigment and cannot be released from their follicles. These immature females will also lack the secondary sexual character of cœlomic cilia. Females with body length in excess of 130 mm. may be suspected of having mature gonads and hence would be susceptible to anterior pituitary-induced ovulation. The normal breeding season is June and July, a single female giving upwards of 5,000 eggs. Females which lay their eggs early in the season will build up another set of eggs by the end of August, making the frogs susceptible to a second induced ovulation during a single year. This may be due in part to the fact that the bullfrog is a ravenous eater, devouring everything from insects and crustacea to small frogs and turtles. Natural ovulation comes just before the most prolific eating period and the recently emptied ovaries immediately start to build up the eggs for the following season.

3. *Rana pipiens* (Schreiber), the leopard frog. The breeding season of this species depends upon the latitude and ranges from the first of April to the middle of May, more than 3,000 eggs being layed by a single female. Possibly because these frogs are not such ravenous eaters as the bullfrogs, a second batch of eggs is not available until the last of August or the first of September.

4. *Rana palustris* (Le Conte), the pickerel frog. The breeding season of this species runs parallel with that of *Rana pipiens* (April 15–May 15) and a single female may lay upwards of 2,000 eggs. While the eggs are the same size as those of *Rana pipiens,* they are bright yellow or brown in color.

5. *Bufo fowleri* (Hinckley), Fowler's toad. This species has been accorded wide distribution probably because it is so easily confused with *B. americanus.* It is found in great and concentrated numbers near the Coast Guard Station on Cuttyhunk Island, Mass. It requires very little water and is sometimes found buried several inches in the hot sand or hopping rapidly through the grass and weeds of the semi-marshy region. The males are distinguished from the females by the black chin. The breeding season is very long, from about the middle of April to the middle of August and occasionally as many as 6,000 or even 8,000 eggs are laid by a single female.

These Anura were collected in their respective habitats and were used within a few days after they were brought to the laboratory. In general the method used for inducing ovulation was similar to that recently described (Rugh, 1934), namely by the injection [1] of fresh anuran anterior pituitary directly into the abdominal cavity. The mammalian extracts used (whole sheep pituitary and antuitrin-S from pregnancy urine) were supplied through the courtesy of the Parke, Davis & Co.

Experimental Data

More than 150 frogs and 200 toads of the various species were used in the experiments tabulated below. With one exception, each tabulation represents an experiment repeated successfully at least four times. That single exception was induced ovulation in *Rana catesbiana* by using antuitrin-S where a single case (the only one tried) reacted favorably. A single male of the same species reacted to 400 rat units of antuitrin-S by attempting amplexus with a female bullfrog. Additional frogs were not available at the time to repeat and confirm these observations.

Attempts were made to induce inter-generic amplexus, using *Bufo fowleri* and the various species of *Rana. Rana pipiens* and *Rana palus-*

[1] The anterior pituitary is not broken up but is injected whole through a large hypodermic, being suspended either in distilled water or 10 per cent alcohol.

tris were not susceptible to sexual stimulation during the summer period but their pituitaries were used to successfully induce sexual reactions in other Anura. It was also found impossible to induce males of *R. clamitans* and *R. catesbiana* to attempt amplexus with any other than their own species. It has been common experience that male frogs show a great deal more discrimination in their sexual reactions than do the male toads.

Toads react very quickly to sexual stimulation, and contrary to the situation among the frogs, they are readily susceptible to mammalian extracts of the anterior pituitary hormone. Not only will the female ovulate with antuitrin-S or whole sheep extract but the male will react to these or to any anuran anterior pituitary by attempting amplexus with almost any object that comes within its range.

If a male toad is injected with two male toad anterior pituitaries, and a female with two female pituitaries, amplexus will be induced within 20 hours and often within 4 hours. Such amplexus will be maintained until the eggs are released through the cloaca, and this has been found to be the best method by which to secure developing toad eggs. (Photo 1.) If whole sheep extract (1 cc.) is injected into a male toad and no females are available (either toads or frogs), amplexus will be induced within 9 hours between male and male. (Photo 2.) If a male toad is injected with 0.5–1.00 cc. of antuitrin-S and is placed alone with a female frog (*R. clamitans, R. pipiens,* or *R. palustris*) amplexus will be attempted within 6 hours and often will be successfully maintained for a period of several days. In one case only a male toad attempted amplexus with a female bullfrog, *R. catesbiana.* These cases of inter-generic amplexus always show the male toad in rather an abnormal position. This is to be expected because of the relative sizes of the frog and toad and the very slippery nature of the frog skin. Usually the toad secures a pectoral grip (Photo 3) but occasionally a pelvic grip (Photo 4) is secured and is found to be more firm and permanent.

Inter-generic amplexus has not thus far resulted in successful cross-fertilization. Undoubtedly the male in amplexus emits spermatozoa but numerous unsuccessful attempts at artificial cross-insemination point toward more complex factors which prevent this situation. Thus far the only successful crosses have been between *Rana palustris* eggs and *Rana pipiens* sperm, in which case the tadpoles were kept for thirty days. Further investigation in this direction is in progress.

Discussion

It is quite evident that the sex-stimulating factor of the anterior pituitary is neither sex nor species specific since mammalian extracts

can be used to induce either ovulation or amplexus in the Anura. The frogs, and to some extent the toads, usually show convulsive reactions following the injection of these mammalian extracts but this may be due to the phenol preservative, or the tricresol or other substances used

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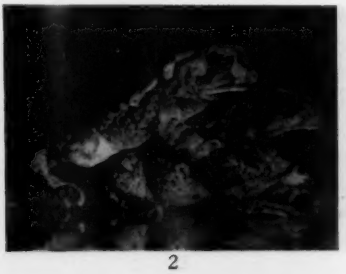

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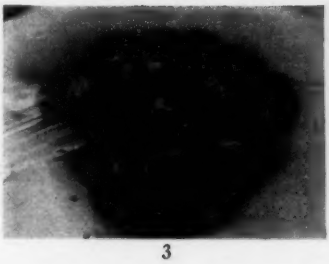

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1. *Bufo fowleri,* male and female in normal amplexus resulting from the injection of standard dose of *B. fowleri* anterior pituitary hormone. Eggs layed in 20 hours, fertilized.

2. Male *B. fowleri,* injected with 1 cc. of extract of whole sheep pituitary shown in amplexus with an uninjected male. Amplexus achieved within 9 hours and maintained for more than 24 hours. Note black chin of ventral male.

3. Pectoral grip of male toad (*B. fowleri*) in amplexus with female green frog (*R. clamitans*) induced by injecting the toad with 1 cc. (100 rat units) of antuitrin-S from pregnancy urine (human). Female not injected. Amplexus maintained for 8–10 hours.

4. Pelvic grip of male toad (*B. fowleri*) in amplexus with female green frog (*R. clamitans*) induced by the injection of antuitrin-S as in Case No. 3. This grip is the more permanent, one pair maintaining amplexus for three days.

(*Note:* These photographs were taken by a Leica Camera with the aid of Mr. E. P. Little of the staff of the Marine Biological Laboratory.)

in extraction rather than to the anterior pituitary hormone from the mammal. As soon as the male overcomes these convulsive reactions it attempts amplexus. Further investigation should be directed along the line of extraction technique so that the pure and concentrated hormone may be available without any harmful by-products. When such

an extract is available, it is entirely possible that the toad (or frog) may be substituted for the more expensive rabbit in human pregnancy tests.

Anuran anterior pituitaries have been found to be uniformly effective in inducing sexual reactions in any anuran providing, of course, that the injected individual has not just gone through a natural breeding season. In no case has induced sexual response appeared in any way abnormal or has it been accompanied by the convulsive reactions which have been associated with the injection of mammalian extracts of the anterior pituitary.

The dose of anuran anterior pituitary necessary to induce the sexual response is, in general, the injection of twice the amount of the host. However, the pituitaries are not uniformly potent for the various species, nor are the relationship of male to female potencies uniform throughout the group of Anura studied. For this reason the data is given in tabular form. As would be expected, the bullfrog gland is relatively the most potent, but this may be only a quantitative relationship since it is the largest of the anuran glands studied. The toad pituitary was found to be the least potent, a fact correlated with its relative size.

It must be remembered that ovulation induced by the injection of the anterior pituitary hormone is not necessarily an all-or-none reaction. The degree to which the ovaries, in a particular case, are emptied, depends upon the dose of the hormone injected and the time of ovulation depends upon the temperature (Rugh, 1935). Taking advantage of these facts, it is possible to so regulate the dose and temperature that the ovaries may be partially (or completely) emptied at a certain specified time. The fact that three of these species can be used to supply eggs (and tadpoles) during the summer months, and *R. pipiens* and *R. palustris* and many others can be so used during the winter months, makes it possible now to say that embryologists need not be without anuran material at any time.

With *R. clamitans* and *R. catesbiana* fertilized eggs can be secured either by inducing amplexus and allowing the pair to lay the eggs in an aquarium or by artificial insemination (Rugh, 1934). The latter method is to be preferred only because it can be controlled and the exact time of fertilization can be ascertained. If care is taken, one can often achieve 100 per cent fertilization by such means. In the case of the toad, *Bufo fowleri,* the situation is quite different. The eggs are not retained in the uterus as they are in the frogs but are layed single (or double) file as they pass through the cloaca. Thus far it has been impossible to have a female toad discharge its eggs into an aquarium containing concentrated sperm suspension and get the eggs fertilized.

TABLE I

*Induced ovulation in Anura during the summer; * June 15 to August 15.*

Form	Breeding Season	Source Anterior Pituitary Hormone												Remarks	Time between injection of hormone and appearance of eggs in uterus, 22–25° C.
		Rana clamitans		Rana catesbiana		Rana pipiens		Rana palustris		Bufo fowleri		Antuitrin S	Whole sheep		
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂				*hours*
Rana clamitans..	June–Aug. 15	2	4	1	1	2	4	2	4	4	6	x	x	Normal fertilized eggs most easily obtained.	17–40
Rana catesbiana.	June–July	4	8			4	8					4 cc. 2 cc. twice daily	x	Produces tremendous numbers of eggs. Single case for mammalian hormone.	40–48
Rana pipiens...	April 1–May 15	x	x	x	x	x	x	x	x	x	x	x	x	Too soon.	
Rana palustris..	April 15–May 15	x	x	x	x	x	x	x	x	x	x	x	x	Too soon.	
Bufo fowleri....	Apr. 15–Aug. 15	2	4	1	1	2	4	2	4	1	2	1.5 cc. .5 cc. twice daily	1 cc.	Most susceptible to induced sexual reactions, both male and female.	7–24

* x denotes failure in reaction.

The only method by which a large percentage of normally fertilized toad eggs can be secured is by inducing amplexus and ovulation simultaneously.

Inter-generic amplexus has been successful only in the one direction illustrated. The toad's skin contains numerous glands, poison to frogs, and this fact alone is adequate to explain the negative results when a male frog is injected in an attempt to induce it to go into amplexus with a female toad. The male toad, however, is very responsive to sexual

TABLE II

Pituitary Induced Amplexus

Form	Source Pituitary Hormone	Reaction Time*
R. clamitans....	1 ♀ *R. clamitans*	No amplexus, inadequate dose
	1 ♀ + 1 ♂ *R. clamitans*	Amplexus in 18 hours
	1 ♀ + 2 ♂ *R. clamitans*	Amplexus 6–24 hours
	2 ♀ *R. pipiens*	Amplexus 6–24 hours
	3 ♂ *R. pipiens*	Amplexus 18–24 hours
R. catesbiana...	400 rat units antuitrin-S (Parke, Davis & Co.)	Brief amplexus (one case only)
B. fowleri.....	1 ♂ or 2 ♂ *B. fowleri*	Amplexus in 2–20 hours
	1 ♀ *B. fowleri*	Amplexus in 2–20 hours
	1 cc. whole sheep extract	Amplexus 5–9 hours
	0.5–1.0 cc. antuitrin-S	Amplexus 2–6 hours

* Reaction time refers to time between injection of the hormone and amplexus. The duration of amplexus is usually several days or until the eggs are extruded from the uterus. In all cases where *Rana* male is in amplexus with *Rana* female, it has been found that the female was also sexually active. This is not necessary with *Bufo*.

stimulation and, when injected with the anterior pituitary hormone, will go into amplexus with almost any available object. By amplexus is meant, not merely a temporary gripping by the forelimbs, but the maintenance of the normal amplectic position for at least two hours. In only two out of eighteen cases of inter-generic amplexus has the female frog been found dead, and these cases might not have been due to toad poison. The male toad, in contrast with the male frog, is rather indiscriminate in respect to the selection of an object for amplexus.

CONCLUSIONS

The doses of anuran anterior pituitary hormone necessary to induce sexual reactions in *R. clamitans, R. catesbiana* and *B. fowleri* during the summer months is given.

Methods are described for the securing of fertilized anuran eggs. It is now possible to secure anuran eggs and tadpoles at any season.

Mammalian extracts of the anterior pituitary (whole sheep gland or antuitrin-S from pregnancy urine) are effective in toads but not uniformly effective in inducing sexual reactions in frogs. It is suggested that this may be due to extraction technique, the by-products of which may be actually harmful to the more sensitive frogs. When the technique is further refined it is possible that the Anura may be substituted for rabbits in human pregnancy tests.

The male toad, *Bufo fowleri*, is shown to be indiscriminate when sexually aroused, going into amplexus with male toads or female frogs of various species when female toads are not available. This is not the case with frogs, where discrimination is evident.

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THE INFLUENCE OF PANTOTHENIC ACID ON GROWTH OF PROTOZOA

ALFRED M. ELLIOTT

(From the Biological Laboratories, University College, New York University, and the College of the City of New York)

Introduction

During the past few years Williams and his associates have been concerned with isolating from many sources substances which stimulate the multiplication of yeast. These investigations finally have resulted in the isolation of a substance to which the name, "pantothenic acid" has been given (Williams et al., 1933). This is an appropriate name because the acid has been found in tissues of representatives of nearly all the plant and animal phyla. It would seem that a substance of such universal occurrence must possess some fundamental biological significance.

Pantothenic acid exerts a pronounced stimulating effect on the growth of the "Gebrüde Mayer" strain of *Saccharomyces cerevisiæ*. Furthermore, it has many properties in common with vitamin G (B_2). Such striking characteristics make it an ideal substance for experimentation with other microörganisms, especially with certain Protozoa because they are more closely related to higher animals than are yeasts. Results of such investigations would be particularly interesting if pantothenic acid should prove to be identical with vitamin G (B_2). Reports in the literature concerning the effects of such substances on the growth of bacteria-free strains of Protozoa are completely lacking, and any evidence to show that Protozoa do or do not require vitamins in their normal nutrition would be of general as well as special interest.

The writer wishes to express his appreciation to Professor R. J. Williams for supplying the pantothenic acid, and also to Miss E. Swaine for counting the organisms in making the tests.

Material and Methods

In testing the effect of such a substance as pantothenic acid on the growth of a protozoan form, it is imperative that the cultures tested are free from other contaminating microörganisms. For that reason all the test cultures used in this investigation are bacteria-free. It was decided to test the effect of pantothenic acid on the growth of a ciliate, *Colpidium striatum*, and a phytomonad flagellate, *Hæmatococcus pluvi-*

alis. The first organism is definitely animal-like in its food requirements while the latter is plant-like in its nutrition. A comparative study was thus conducted with two remotely related protozoan forms, one definitely animal-like, the other plant-like.

The bacteria-free strain of *Colpidium striatum* employed was the one used in a previous investigation (Elliott, 1933). The pure strain of *Hæmatococcus pluvialis* (bacteria-free) was originally obtained from Professor E. G. Pringsheim, and was used recently in a morphological study (Elliott, 1934). Professor R. J. Williams very kindly supplied the pantothenic acid in the form of a calcium salt which had a potency of "244"; "one milligram added to 2.5 liters of culture medium gave a heavy response with yeast in 18 hours." Difco tryptone was employed in an organic basic culture medium, which was made up as follows:

Difco tryptone	10.0 gram
KH_2PO_4	2.0 "
Distilled water	1.0 liter

This medium was used throughout the experiments with *C. striatum*, while with *H. pluvialis* the tryptone content was reduced to 0.5 per cent because previous experiments had shown that the flagellate grew better in such a concentration.

For experimental purposes large quantities of the basic medium were made up and subsequently divided into two parts; to one portion was added HCl in titrating for the lower pH values and NaOH was added to the other portion in titrating for the higher pH values. Usually 10 tubes were set at each desired pH unit, each one of which contained 8 cc. of basic medium; to 5 of these (after sterilization) 1.0 cc. of a sterile pantothenic acid solution (10 mg. in 250 cc. of distilled water) was added. To the other 5 tubes distilled water (1 cc.) was added. The tubes were then inoculated with organisms from a stock culture prepared in the following manner. A heavily growing culture tube of the organisms was pipetted into a sterile centrifuge tube and centrifugalized. This was repeated at least four times with sterile tap water. Finally the washed organisms were pipetted into a dilution flask containing 200 cc. of sterile tap water. From this flask 1-cc. inoculations were made into each tube; the flask was vigorously shaken before each inoculation in order to insure an even distribution of the organisms. One tube was then selected from each set and tested for the initial pH. Most of the series were incubated at room temperature (19–26° C.) for a period of 76 hours or longer. After the incubation period was completed, two or more of the tubes from each set were checked for the final pH. One-half cc. of Bouin's fixative was then added to these and the remaining tubes and the final counts made; this was done with

the aid of a Sedgwick-Rafter counting chamber and a Whipple ocular micrometer. A LaMotte roulette comparator was employed in making pH determinations.

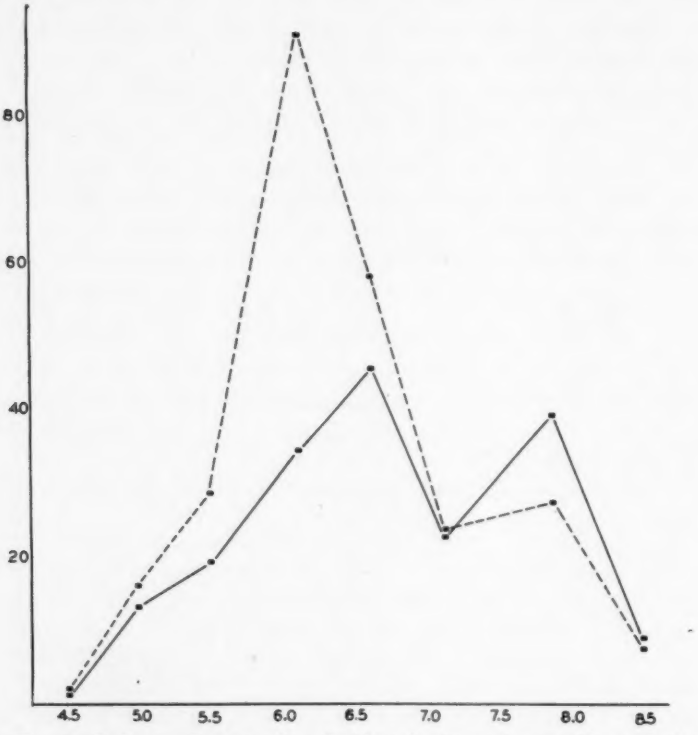

FIG. 1. Series I. Concentration of ciliates in thousands per cubic centimeter plotted against initial pH. The solid line indicates growth in the control; the broken line, growth with pantothenic acid.

EXPERIMENTAL

In testing the effect of any ionizable substance such as pantothenic acid, it is essential to determine its influence over a wide pH range, and not at the point of optimum growth alone of the organism. A substance may have decidedly different effects in the ionized and molecular condition. This fact was well illustrated in the case of acetic and butyric acid (Elliott, 1933a); these two substances were definitely toxic in the lower pH ranges (6.5–4.5), while above pH 7.0 they actually accelerated growth of *Colpidium striatum* and *C. campylum*. For this reason pantothenic acid was tested over a wide pH range.

Series I—Colpidium striatum

The pH values were set at 4.5, 5.0, 5.5, 6.3, 6.6, 7.2, 7.9, and 8.5. After sterilization and inoculation the pH remained the same in all the tubes. After 80 hours of incubation at room temperature there were no pH changes, except in the tubes set at pH 6.3 and there the change was within the experimental error of the readings ($\pm$ 0.1). The final counts are recorded in Fig. 1.

It is obvious that a marked acceleration occurred in the tubes containing pantothenic acid over those of the controls. A noticeable increase was observed at pH 5.5, with a maximum at 6.3 and 6.6; there was no traceable increase at 7.2 and actual deceleration above this point. Pantothenic acid, as evidenced by this series, accelerates growth of *Colpidium striatum* in the acid range but has no effect in the alkaline range. The bimaximal pH growth curve in the control is typical for this species (Elliott, 1933a).

This entire series was duplicated (Series Ia) with similar results.

Series II—Hæmatococcus pluvialis

The experimental procedure in this series was very similar to that in Series I. The tubes were set at the following pH units: 4.5, 5.0, 5.5, 5.9, 6.5, 7.0, 7.5, 8.2, and 8.5. After 96 hours of incubation at room temperatures and north window illumination there were no pH shifts. The final counts are recorded in Fig. 2.

The results indicate that pantothenic acid had no effect whatever on the growth of *Hæmatococcus pluvialis* over the entire pH range. The differences at pH 7.0 and 8.2 are probably insignificant. It appears then that this substance accelerates the growth of a saprozoic ciliate, yet has no effect on a chlorophyll-bearing flagellate.

Series III—Colpidium striatum

It was shown in a previous investigation (Elliott, 1933b) that gelatin would not support growth of *Colpidium* beyond the third transfer. Gelatin lacks certain amino acids (tryptophane, isoleucine, and hydroxy glutamic acid) but is rich in lysine which is an essential amino acid for the growth of this organism (Hall and Elliott, MSS). Some other substance is apparently lacking in this protein. It occurred to the writer that perhaps pantothenic acid might be such an essential missing substance. For that reason it was thought worth while to determine the effect of pantothenic acid on the growth of *Colpidium striatum* when gelatin was employed as a basic protein. The experimental procedure was very similar in this series to that in the previous two with the ex-

ception that Difco gelatin (1 per cent solution) was substituted for Difco tryptone in the basic medium. The pH was adjusted as before at the following values: 4.5, 5.5, 6.5, 7.0, 7.5, 8.0, and 8.5. Pantothenic acid was added to one half of the tubes, sterilized, inoculated, incubated and counted. The final determinations are recorded in Fig. 3.

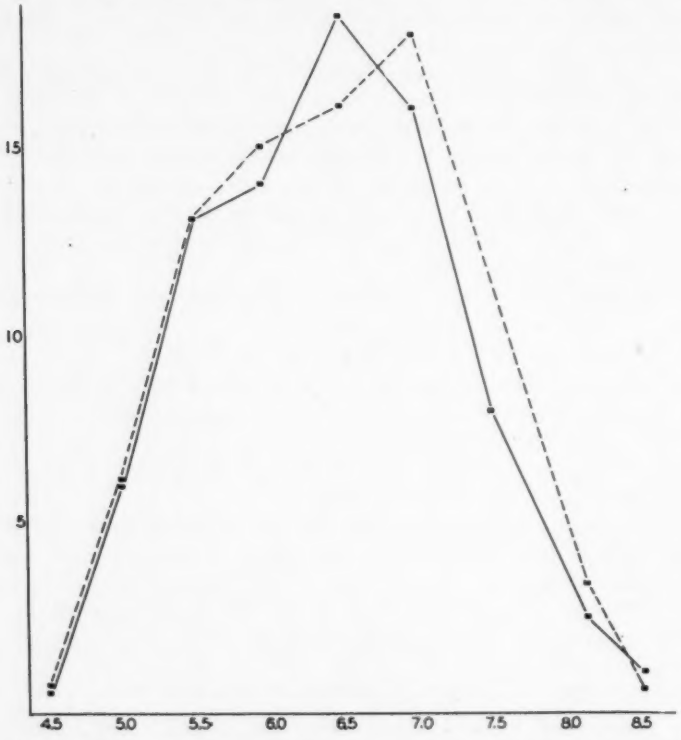

FIG. 2. Series II. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. The solid line indicates growth in the control; the broken line, growth with pantothenic acid.

The slight growth which occurred in the control tubes was probably due to substances carried over with the original inocula; the stock organisms were not washed as in the previous series. For that reason a small amount of growth would be expected. Had the washings and dilutions been made, no growth would probably have resulted. The essential point, however, is that pantothenic acid is apparently not the substance lacking in gelatin for the support of the growth of *Colpidium*.

Some other, still unknown, substance is deficient in this incomplete protein.

Series IV—Colpidium striatum

It was shown in a previous investigation (Hall and Elliott, MSS) that by adding small quantities of yeast extract to gliadin that growth could be maintained indefinitely; likewise, by adding a small amount of yeast extract to zein, together with lysine which is lacking in this protein, indefinite growth could be maintained. This indicated that some substance in yeast was essential for the growth of *Colpidium*. Perhaps this substance was pantothenic acid? The following series of experiments was devised to test this point.

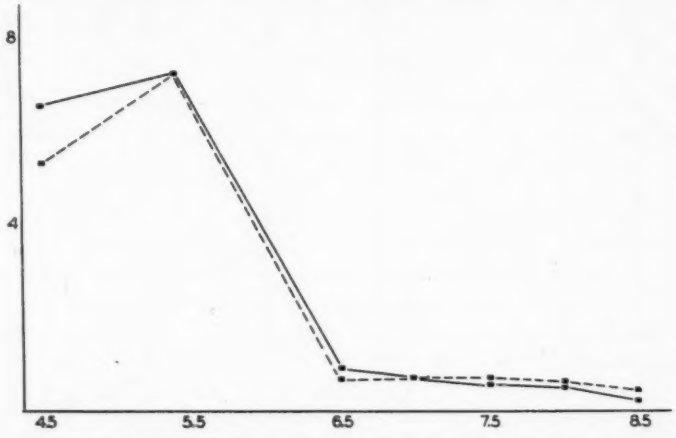

FIG. 3. Series III. Concentration of ciliates in thousands per cubic centimeter plotted against pH. The solid line indicates growth in the gelatin control; the broken line, growth with pantothenic acid.

The procedure was slightly different from that employed in the foregoing series. The basic medium consisted of 1 per cent protein (zein, gliadin, or gelatin) and 0.2 per cent KH_2PO_4 in distilled water. The pH was set at 5.8 in all tubes since previous experiments had demonstrated that acceleration occurred in that range. To 6 tubes containing zein (8 cc. each) lysine was added (1 cc. of 0.7 per cent stock solution); to each of 6 others, was added 1 cc. of pantothenic acid (concentration identical with that used in Series I); and to a third set of 6 tubes was added both lysine and pantothenic acid. In a similar manner lysine and pantothenic acid were added to 3 sets (6 each) of tubes

containing gliadin. In this case, however, lysine was added because the concentration of this amino acid in gliadin is low (0.92 per cent). Gelatin was included in this series merely as a check on the previous

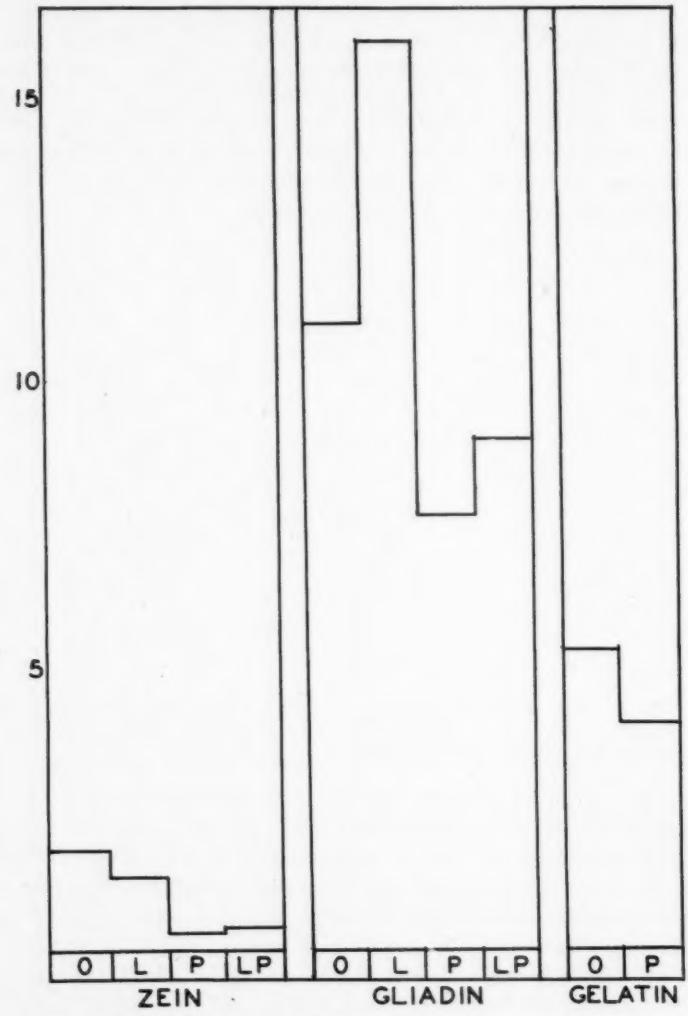

FIG. 4. Series IV. Concentration of ciliates in thousands per cubic centimeter plotted on ordinate. The letters in each column indicate substances added: *O*, nothing; *L*, lysine; *P*, pantothenic acid; *LP*, both lysine and pantothenic acid.

observations. All the tubes were inoculated from a stock culture which had not been washed so that a small amount of the original medium was carried over into each tube. This would permit a small amount of growth even if the tested substance was inadequate to support continued growth. The incubation period was 80 hours and the final counts are recorded in Fig. 4.

It is observed at once that in the case of all three substances, zein, gliadin, and gelatin, pantothenic acid had no accelerating effect. In fact, an actual deceleration is noted in all three cases, although perhaps significant only in the case of gliadin. The growth was comparatively slight in all tubes, which was to be expected if pantothenic acid was ineffective. It may be concluded, therefore, that pantothenic acid is not the substance essential for life of *Colpidium* which is lacking in zein, gliadin, and gelatin.

Discussion

The question of growth-promoting substances in microörganisms has commanded considerable attention for many years. It was initiated by Wildiers (1901), who isolated his so-called "bios" from yeast, and who maintained that this substance was essential for continued growth of the organism. An excellent review of the history of the study of bios is given by Tanner (1925). Fulmer and Nelson (1922) recognized the significant increase of yeast growth by bios but found that it is not necessary for indefinite growth of the cells. Williams (1919) began a long series of experiments which finally resulted in his isolation of pantothenic acid (Williams et al., 1933) which he and his associates claim is identical with, or very closely related to, vitamin G (B_2). Several of its properties suggest such a relationship. It probably is one of the substances originally contained in Wildier's bios. Williams believes that the accelerating substance in the growth of yeast is a single substance, namely, pantothenic acid. Richards (1932), on the other hand, believes that thallium, found as an impurity in asparagin and many other sources, may be the element responsible for acceleration when used in proper concentrations. The striking effect of pantothenic acid on the growth of *Saccharomyces cerevisiæ*, as demonstrated by Williams and his associates (1933), seems more impressive than the results obtained by Richards with thallium. The former workers showed that concentrations as low as 0.02 mg. in 1 cc. of their synthetic culture medium was sufficient to accelerate the growth five times that of the control. Increasing concentrations produced corresponding increases in the growth rate; when the concentration reached 40.0 mg. per cc. of culture medium the fold increase was about 1500 times that of the

control. The very high dilutions preclude the possibility of pantothenic acid being utilized as a source of food. Therefore, its activity in the organism probably simulates that of vitamins in higher forms.

Although the effect on Protozoa is not so striking as that produced with yeast, nevertheless the present experiments indicate that the effect of pantothenic acid on the growth of *Colpidium striatum* at certain pH values is very pronounced. The increase is definite on the acid side of neutrality, while above pH 7.0 no acceleration occurred. It was at first thought that this might be due to the toxic effect of the dissociated acid (ionization constant is approximately 3.9×10^{-5} according to Williams and Moser, 1934). However, upon examination of the properties of the acid, it was found that according to Williams it is thermo-labile in the alkaline range. They say, "It is evident that nearly all of its (pantothenic acid) activity . . . is destroyed by prolonged heating in weakly alkaline medium but that heating in weakly acid or neutral medium for 4 hours at 119° C. causes very little destruction." The destruction of its accelerating powers on the alkaline side in Series I might have been due to the heating to 122° C. for 20 minutes when the tubes were sterilized.

The question of synthesis of pantothenic acid by plants and animals was studied by Williams and his associates (Williams et al., 1933). They found that *Aspergillus niger* synthesized the acid in soils and this led them to believe that it was not produced by green plants. They say, "that it is without doubt produced by microörganisms in soil suggests the possibility that it may not be synthesized by green plants." This prediction is of interest in view of the present observations. It was noted that the green phytomonad, *Hæmatococcus pluvialis*, failed to show any acceleration with pantothenic acid while a marked increase was noted with the saprozoöic organism, *Colpidium striatum*. This does not prove that *Hæmatococcus pluvialis* fails to synthesize the acid but it does seem to indicate that the organism does not utilize it to any great extent.

It is not at all unusual that microörganisms are able to synthesize vitamins; for example, Kuroya and Hosaya (1923) showed that *Bacterium coli* synthesized vitamin B, and Damon (1924) demonstrated that several members of the genus *Mycobacterium* produced a growth-stimulating substance analogous to vitamin B. It appears that this ability to synthesize vitamin B is widespread among bacteria and yeasts. Vitamin G (B_2) is probably synthesized along with B since it has somewhat similar distribution and properties. Both vitamin B and G were probably present in the Difco tryptone base (partially hydrolized casein) used in the present experiments, but since all the tubes were autoclaved

at one time or another, properties of G were undoubtedly impaired. Furthermore, the writer has maintained bacteria-free cultures of Protozoa for several years in autoclaved media. Since vitamin B is destroyed by such treatment, it is probably not essential for the growth of these organisms. On the other hand, vitamin G (B_2), being heat stable under most conditions, probably was retained unharmed for the most part and possibly utilized by the ciliates. If vitamin G and pantothenic acid prove to be identical substances the results of the present investigation will carry more interest. While these observations do not demonstrate the essential nature of pantothenic acid in regard to continued culturing of *Colpidium siriatum*, they do indicate its importance in rate of multiplication of the ciliate.

Summary

Pantothenic acid, a growth-promoting substance of universal occurrence, was tested for its effect on the growth of two remotely related protozoan forms, namely, *Colpidium striatum* and *Hæmatococcus pluvialis*. The tests were conducted over a wide pH range. In the case of *C. striatum* a doubling of the growth occurred in the pantothenic acid cultures on the acid side of neutrality (pH 5.5–6.6) while no acceleration was observed above pH 7.0. With *Hæmatococcus pluvialis* no differences were noted in the test and the control tubes. It was shown further that pantothenic acid was not the substance lacking in certain incomplete proteins (zein, gliadin, and gelatin) which failed to support indefinite growth of *C. striatum*.

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THE LETHAL ACTION OF SUNLIGHT UPON BACTERIA IN SEA WATER

CLAUDE E. ZOBELL AND GEORGE F. MCEWEN

(From the Scripps Institution of Oceanography, University of California, La Jolla, California)

Although our knowledge of the distribution and activities of marine bacteria is woefully scant, it is recognized that these microörganisms play an important rôle in the biology, geology, and chemistry of the sea (cf. Bavendamm, 1932; Benecke, 1933; and Waksman, 1934). There are several factors which are known to influence the distribution of bacteria in the sea, but so complex is the problem that it is necessary to consider each factor singly in order to evaluate its significance.

It has been stated frequently that the lethal action of sunlight upon bacteria has a profound effect upon their vertical, diurnal, and seasonal distribution. For example, Fischer (1894) found more bacteria in the North Atlantic at sunrise than in the afternoon, and Schmidt-Nielson (1901) attributed to the killing action of sunlight the difference between 26 bacteria per cubic centimeter of surface sea water and 420 per cubic centimeter at a depth of 25 meters. Similarly, Bertel (1912) noted that during May and June, a period of intense insolation, the number of bacteria in the Atlantic between the Azores and Portugal increased from the surface downward and, furthermore, the number of bacteria at the surface increased during the night and was reduced again in the morning. Gaarder and Spärck (1931) ascribed to the bactericidal effect of sunshine the paucity of bacteria in Norwegian oyster pools in the summer as compared to their greater abundance in the winter. Corresponding observations have been made in fresh-water lakes as exemplified by the report of Graham and Young (1934), who found the maximum bacterial population at a depth of 30 to 60 feet, stating that there were fewer bacteria near the surface due to the intensity of the light. Conversely, Reuszer (1933) found no correlation between the numbers of bacteria in surface sea water and the time of exposure to sunshine in the summer months near Cape Cod. Fred, Wilson, and Davenport (1924) found no evidence of light influencing the bacteria in the surface water of Lake Mendota. Lloyd (1930) observed that although the number of bacteria in the surface layers of water in the Clyde Sea area increased slightly during the hours of darkness, even on sunny summer days there were more bacteria at the sur-

face than in the underlying strata, and she deduced that the bactericidal effect of sunlight is negligible. In their studies on the factors which influence the distribution of bacteria in the sea, ZoBell and Feltham (1934) concluded that if there is any direct or indirect harmful effect of sunlight, it is obscured by other factors. This paper is a continuation of those studies.

Experimental

Observations on the vertical distribution of bacteria were made on several bright calm days during the summer months of 1933 and 1934 at the end of the Scripps Institution pier which extends 1,000 feet into the sea and beyond the surf zone. The sampling device consisted of a sterile evacuated bottle fitted with a sealed capillary tube which is broken

Table I

Average plate counts expressed as bacteria per cubic centimeter of sea water collected at various depths.

Depth	Date of experiment		
	6/5/1934	7/2/1934	8/3/1934
meters			
Surface	87	206	48
0.1	62	249	78
0.5	38	164	35
1.0	80	91	73
2.5	94	134	42
5.0	63	282	27

by a messenger, thus permitting the bottle to fill with uncontaminated water from any level within a few centimeters of the desired depth. For a complete description of the sampling device and details concerning the methods of investigation, evaluation of the experimental error, and the lack of uniformity in the distribution of bacteria in the sea, see ZoBell and Feltham (1934).

Samples consisting of 50 cc. of sea water were collected at 2:00 P.M. from the surface and at various depths below the surface. After vigorously shaking the samples, appropriate dilutions thereof were plated in duplicate on nutrient sea-water agar. The plates were incubated for four days at 25° C., and the resulting colonies were enumerated in a Stewart counting chamber, using a 3.5x engraver's lens. The average number of bacteria per cubic centimeter are shown in Table I.

This experimental procedure presented no evidence that sunlight destroyed the bacteria in the upper strata of water for, within the limits

of sampling errors, there were just as many bacteria in the surface water as in that taken from a depth of 5 meters. These results corroborate previous findings when the mean of twelve samples collected from August 1 to August 13, 1932, was 584 bacteria per cubic centimeter of surface sea water, 447 in the 3-meter strata, and 608 from a depth of 6 meters. It is recognized that the vertical circulation of the water near shore may have a tendency to mix the different strata, but that mixing was not appreciable during this period is indicated by a temperature difference of from 2° to 6° C. between the surface and bottom water at the end of the pier.

The vertical distribution of bacteria to greater depths was investigated near mid-day during the summer months. On the boat " Scripps "

TABLE II

Vertical distribution of bacteria at sea expressed as the number of bacteria per cubic centimeter of water at different depths.

Depth	Station no. and date			
	1. 9/7	1. 9/14	3. 6/24	3. 7/16
meters				
Surface	147	344	27	165
5	126	400	47	164
10	238	324	22	253
20	292	528	—	406
50	86	620	109	285
100	14	17	31	98
150	2	0	—	—
200	3	2	2	0
300	6	0	5	2
400	0	1	3	—
500	2	0	2	3

bacteriological samples were obtained from intermediate depths to 500 meters at stations ten to fourteen miles offshore where the water was approximately 1,000 meters deep. The results of duplicate analyses of these samples are shown in Table II. The bacterial population was found to increase from the surface downwards to a depth of about 50 meters, which is in accordance with the observations of Føyn and Gran (1928), Schmidt-Nielson (1901), and Bertel (1912).

It should be noted that from the surface to the zone of their greatest abundance the number of bacteria increases only two- to four-fold, which is not indicative of a direct bactericidal effect of solar radiations in the upper strata since the penetration of these rays decreases geometrically with depth. If we assume for the sake of argument that the lethal action of sunlight is the only factor which influences the otherwise uniform

vertical distribution of bacteria throughout the zone of photosynthesis (which is known to be a false assumption), we would expect the number of bacteria to increase in geometric progression from the surface downward following several hours of exposure to intense sunlight. There is no semblance of such a relationship. As is shown in Fig. 2, virtually all of the bactericidal radiations, or those having a wave length of 3130 Å or less, are absorbed by the first three meters of sea water and there is no significant increase in the bacterial population until a depth of ten to twenty meters is reached. While there is no indication from the study of the vertical distribution of bacteria in sea water that sunlight has any lethal action, the experiments fail to prove that there is no such

TABLE III

Average plate counts expressed as bacteria per cubic centimeter of surface sea water collected at different times during the day.

Date of collections	Time of collection		
	7:00 A.M.	12:00 M.	5:00 P.M.
June 4	192	167	80
5	180	158	167
6	109	81	102
7	122	—	216
8	204	239	94
11	51	12	34
12	—	103	143
13	217	141	166
14	165	315	72
15	96	283	158
Average	148	167	123

action because there are multifarious other factors which influence the vertical distribution of bacteria about which little or nothing is known.

DIURNAL AND SEASONAL FLUCTUATION

During a two-week period from June 4 to June 16, 1934, 50-cc. samples of sea water were collected from the end of the pier at 7:00 A.M., 12:00 M., and 5:00 P.M. These samples were analyzed for their viable bacteria content by the same standard procedure described above. Table III presents the findings.

While in some cases there was a perceptible diminution in the number of bacteria per unit of water after being exposed all day to sunlight, on other days the 5:00 P.M. samples actually contained more viable bacteria than the early morning samples. The tidal phase was taken

into consideration, and it did not influence the results. It is recognized that the same mass of water was not being sampled throughout the day, due to its slow horizontal circulation which accounts for many of the apparent discrepancies. This, together with the fact that bacteria are not uniformly distributed in sea water and that there are certain unavoidable experimental errors in the collection and analysis of samples, emphasizes that the formation of conclusions from one day's observations, as has been done by other workers, is unreliable and untenable. However, most of the errors of single observations should be offset by averaging several observations, and the application of this method re-

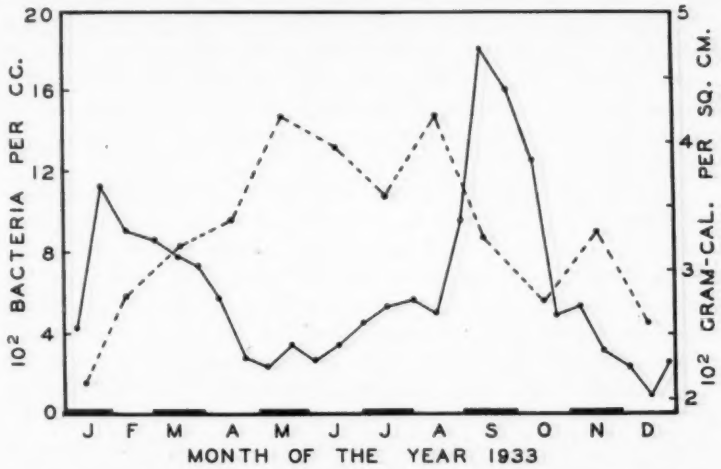

FIG. 1. The solid line gives the bi-weekly average number of bacteria per cubic centimeter of surface sea water during the year 1933 and the broken line gives the corresponding monthly mean solar radiation intensity in terms of gram-calories per square centimeter.

vealed that the bacterial counts of the 5:00 P.M. samples were only slightly lower than the 7:00 A.M. samples and, indeed, the 12:00 M. samples contained more bacteria than the early morning ones. Previously, from August 15 to September 13, 1932, by use of a similar procedure, an average for 28 days showed 487 bacteria per cubic centimeter of surface sea water in the early morning and 455 in the late afternoon.

The seasonal distribution of bacteria in the sea has been followed for three years by the daily (except Sunday) analysis of water samples collected from the Institution pier. Data, showing the relationship be-

tween the bacterial population expressed as bacteria per cubic centimeter of surface sea water and the insolation intensity expressed in terms of gram-calories per square centimeter, are summarized in Fig. 1. Biweekly averages of the former for the year 1933, and the corresponding monthly mean insolation intensity calculated from the daily values recorded by the Institution's pyroheliometer, are given. A description of this instrument appears in the *Monthly Weather Review*, vol. 60: pp. 26–28, 1932, and information on the measurements of solar radiations at La Jolla may be found in subsequent issues.

In Fig. 1 it will be noted that the period of maximum insolation, May to July, was also a period of minimum bacterial population, which from these data alone may be interpreted as a bactericidal effect of sunlight. But it should also be noted that by far the largest number of bacteria occurred in September when the insolation was still relatively intense, and very few bacteria were found the last of November to the first of January when insolation was at its lowest ebb. The same general trends were found in 1932.

Like the foregoing experiments, there are too many additional physical, chemical, and biological factors which also undergo seasonal cycles and which are known to affect the bacterial population of the sea to warrant an evaluation of the direct influence of sunlight alone. For example, it has been found that, except for the large numbers of bacteria which occur each year in February, there is a semblance of correlation between the water temperature and the bacterial population (cf. ZoBell and Feltham, 1934). Also, the phytoplankton content of the water at the same place was highest from May to July, and next highest in November and December, which, if only one factor is considered, may indicate that periods of maximum photosynthesis are accompanied by small bacterial populations and followed by large ones. Thus, while a comparison of the insolation intensity with the seasonal distribution of bacteria indicates that the former may exert a harmful influence on bacteria, the study illustrates the fallacy and futility of trying to interpret findings with insufficient data on contributing factors. Evidence is being rapidly accumulated by field workers that light acts as an important factor in the distribution of both plant and animal species in the sea and the abundance of bacteria is interlinked with the presence of either dead or living organic matter. The vertical distribution of phytoplankton is directly associated with light intensities (Pettersson et al, 1934) and there are numerous phototropic zoöplankton (cf. Spooner, 1933, Gardiner, 1934) which migrate to the surface during hours of darkness and sink to deeper water with increasing luminosity, all of which would affect the vertical, diurnal, and seasonal distribution of bacteria.

Controlled Experiments

Direct field observations having failed to answer satisfactorily the question under consideration, laboratory experiments were devised in which the various conditions were under control. During bright days in July when the angle of incidence of the sun's rays approached 90° at mid-day, shallow layers of sea water were exposed in open Petri dishes on the roof of a two-story building. The sea water was paper-filtered to remove suspended particles and thereby insure a more uniform distribution of bacteria. The dishes of sea water were held in a water bath the temperature of which was maintained at 25° to 26° C. with running tap water. Sea water was placed in the dishes having a diameter of 10 cm. to give depths of 2 mm., 5 mm., and 10 mm., this requiring 16 cc., 40 cc., and 80 cc., respectively. The number of bacteria in the water was determined by plate counts at the beginning of the experiment and at intervals thereafter. The average results of three such experiments are summarized in Table IV.

Table IV

Average number of bacteria per cubic centimeter of sea water of different depths surviving exposure to direct sunlight from 11:00 A.M. to 1:00 P.M.

Time of exposure	Depth of water		
	2 mm.	5 mm.	10 mm.
Initial	164	159	163
15 minutes	109	135	138
30 minutes	93	124	140
1 hour	83	139	127
1½ hours	81	95	122
2 hours	76	108	126

The experiment clearly demonstrates that sunlight does have a lethal action on bacteria suspended in shallow layers of sea water, this action being most marked during the first few minutes of exposure and being almost negligible after one hour. It also indicates that the bactericidal action apparently decreases with depth even within the upper 10 mm. of water. Such shallow layers of water were also exposed to sunlight from 8:00 A.M. until 4:00 P.M., but the diminution in the number of viable bacteria was not much, if any, greater than when exposed to the mid-day sun for one hour. Nor was there any evidence of a cumulative effect following exposure on five successive days as compared with the controls which were kept in the dark at the same temperature.

In order to examine more closely the relationship of depth to the

bactericidal action of sunlight, 38-cm. battery jars were filled with paper-filtered sea water to give a depth of 35 cm. of water. One of these was exposed to bright July sunlight and the other was kept in the dark. Both were maintained at 25° to 26° C. in a bath of running water. Samples were obtained from the surface and at depths of 10, 20, and 35 cm. by means of a sterile pipette, the upper end of which was kept closed until the pipette had been inserted to the desired depth. These samples were collected at 9:00 A.M., 1:00 P.M., 4:00 P.M., and 9:00 P.M., and were plated on nutrient sea water agar. Table V presents the averaged results of three such experiments on three different days.

TABLE V

Average plate counts of sea water in three experiments in battery jars at different depths with and without exposure to sunlight.

Treatment	Depth of sample	Time of taking sample			
		9:00 A.M.	1:00 P.M.	4:00 P.M.	9:00 P.M.
	cm.				
Covered.....	surface	241	202	190	196
Covered.....	10	235	263	188	211
Covered.....	20	228	197	225	208
Covered.....	35	217	204	217	242
Exposed.....	surface	238	152	121	130
Exposed.....	10	246	173	198	164
Exposed.....	20	209	195	176	153
Exposed.....	35	254	211	187	228

Just prior to taking the 9:00 A.M. samples the water in both jars was thoroughly mixed by pouring from one jar into the other, so the difference in the plate counts in the 9:00 A.M. column furnishes an index to the range of error in the analytical procedure. Again it is demonstrated that sunlight kills bacteria in sea water, but it does so to a detectable degree only in the uppermost few centimeters of water, and even in the upper layers the lethal action is relatively slight. From the 4:00 P.M. column in Table V it should be observed that following seven hours exposure to direct sunlight the bacteria in the surface layer were reduced only 36 per cent as compared to the covered control. The average of the four strata at 4:00 P.M. was 205 bacteria in the covered jar and 171 in the exposed jar, and just about the same at 9:00 P.M. Exposure of the water in battery jars on several successive days did not cause any more tangible decrease in the bacterial population than in the unexposed control. In both jars sedimentation and thigmotro-

pism became evident. It has been pointed out by Prescott and Winslow (1931) that the tendency of bacteria to settle in standing water has been misinterpreted as a lethal action of sunlight, and ZoBell and Allen (1933) have showed that many bacteria are thigmotropic and, when confined in glass vessels, attach themselves to solid surfaces.

Although the foregoing results are not always clear-cut, the findings are in perfect accord with our knowledge of the abiotic radiations of

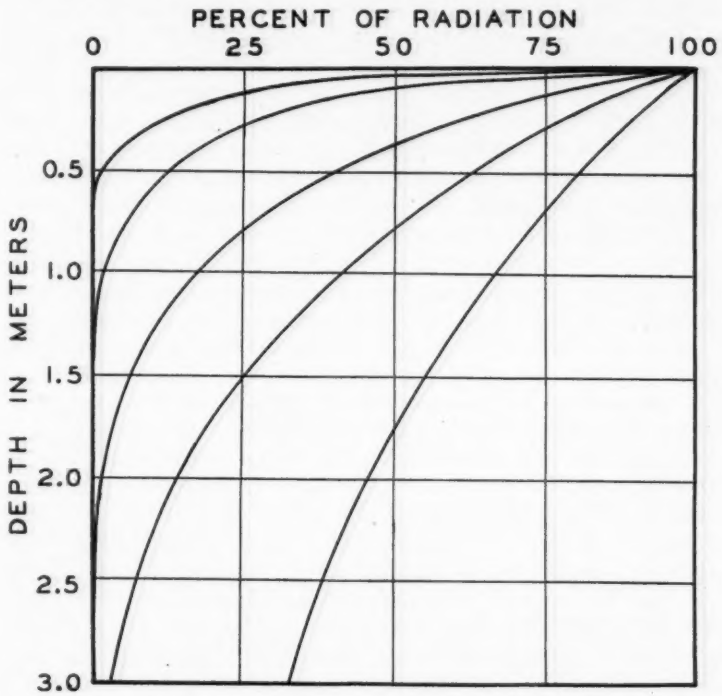

FIG. 2. Percentage of incident intensity of radiations of wave lengths, reading from top to bottom, 2540, 2660, 2800, 3030, and 3340 Å, which penetrate clear sea water to different depths.

sunshine. Recent work by Hulburt (1928), Atkins (1932), Richardson (1932), and others shows that the penetration of light in sea water decreases rapidly as the wave length decreases. This is illustrated by Fig. 2, which gives the depth of penetration of certain bactericidal radiations in terms of the percentage of the incident rays which reaches the stated depth. The data were calculated by using the approved formula and the absorption coefficients found by Hulburt (1928).

From a comprehensive review of the literature, Ellis and Wells (1925) conclude that the bactericidal range of solar radiations is from 2960 Å to 2100 Å, with the maximum between 2800 Å and 2500 Å. This is also the consensus of opinion of the workers whose findings are reviewed by Buchanan and Fulmer (1930). A few investigators claim that wave lengths up to 3660 Å are perceptibly abiotic but, indeed, they are only feebly so, as indicated by the work of Cernovodeanu and Henri (1910), who noted that whereas 3 to 5 hours were required to sterilize a thin emulsion of *Bacterium coli* with wave lengths greater than 3050 Å, it was sterilized in 15 to 20 seconds with shorter wave lengths. Now it will be observed from Fig. 2 that the penetrating power of the radiations of maximum bactericidal action (2800 Å to 2540 Å) is very small, virtually all of these radiations being absorbed by the first meter of sea water and their intensity reduced nearly one-half by passage through only 10 cm. of sea water. The bactericidal action of ultra-violet radiations decreases much more rapidly than the decrease in the intensity, as has been shown by Coblentz and Fulton (1924). Fischer and Holden (1927) found that the lethal action of ultra-violet radiations is determined directly by their intensity and it seems to be a logarithmic function. Furthermore, it should be stated that the data in Fig. 2 are based upon observations in which the angle of incidence of the radiations is 90° and in quiet clear sea water free of suspended matter. The transmission of radiations is reduced proportionately as the angle of incidence decreases and, likewise, when the surface of the water is ruffled by wind or wave action. Also, the penetration is materially less in sea water containing organic matter, especially if the latter is particulate. Grimm and Weldert (1911) found that as few as 100 bacteria per cubic centimeter greatly increased the absorption of ultra-violet rays. Working at sea, Richardson (1932) observed that 21 per cent of the incident rays of sunlight up to 4800 Å is absorbed by the first 5 mm. of sea water, and it will be recalled that these longer wave lengths are far more penetrating than the ultra-violet rays.

Incidentally, it may be of interest to point out that the absorption coefficients of ultra-violet radiations in sea water are much greater than in fresh water. For example, the absorption coefficient as found by Hulburt (1928) for the wave length of 3030 Å is 0.017 in sea water and only 0.005 in distilled water, which means that the incident intensity of this radiation will be reduced to 18 per cent after passing through 10 cm. of sea water, whereas 95 per cent of it will penetrate this depth of distilled water. In natural fresh water Buchner (1893) noted only a feeble lethal action of sunlight on bacteria, the bactericidal power penetrating less than 3 meters, and Jordan (1900) found that in river

water the sun's rays are virtually without action. Topley and Wilson (1928) conclude that even in clear water it is doubtful if abiotic rays are active for a distance of more than 5 feet from the surface and, due to the turbidity and constant movement of water in nature it is improbable if bacteria are subjected to the influence of the rays for sufficient time to kill them.

Conclusions

Observations on the vertical, diurnal, and seasonal distribution of bacteria in the sea fail to show evidence of a lethal action of sunlight. While the seasonal fluctuation in the bacterial population is more or less inversely proportional to the intensity of sunlight, it is recognized that there are multifarious interlinked biological, physical, and chemical factors which also influence the bacterial population of sea water.

Controlled laboratory experiments reveal that at this latitude sunlight has a feeble lethal action on bacteria in the uppermost few millimeters of sea water, but even shallow layers of sea water are not sterilized by prolonged exposure.

Virtually no bactericidal radiations penetrate sea water three meters, and the intensity is materially reduced by passage through 10 cm. of sea water.

Appendix

While the data summarized in Table V indicate that sunlight does kill bacteria in sea water, there is an unavoidable residual error or variability of the plate counts even when conditions are held as constant as possible. Therefore, in order to determine details regarding quantitative relations, and to estimate their statistical significance, the following method of analysis was selected as being appropriate:

Assume the variability of the counts with respect to time to be independent of the depth. Determine the statistical significance of differences between the variabilities at different depths using, as is customary, the standard deviation, or S.D., for an index of the variability. Attribute to changes in the controlling factor, radiation, any differences that could not reasonably arise as a result of the sampling or residual error. In order to eliminate the effect of differences in the initial counts of the three experiments, all results of each experiment were expressed in the percentage of the average count at all levels for that experiment at the initial time, 9:00 A.M. The average results, including the S.D. but not the individual counts, are presented in Table VI. Lethal action of sunlight is indicated by the regular decrease of the S.D. of values exposed. On account of the small number or size of sample in each column, tests of significance should be made in accordance with theories

of small samples, for example, that of Fisher (1928). There are twelve individual observations per column of individual counts corresponding to eleven "degrees of freedom," and, according to Fisher's Table VI, the natural logarithm of the ratio of any two S.D.'s must exceed 0.52 for chances of less than 5 in 100, and exceed 0.75 for chances of less than 1 in 100. Accordingly, comparing the S.D., 25.6 of surface values exposed with the S.D., 14.5 of bottom values, the chances are about 4 in 100. Comparisons of any two other values of the S.D. corresponding to the exposed jar indicates chances exceeding 5 in 100. Comparisons between the S.D., 25.6, of surface values exposed with the S.D., 10.2, of surface values covered, gives a chance less than 1 in 100. The difference is not significant at the 10-cm. level

TABLE VI

Average plate counts of sea water in three experiments in battery jars at different depths with and without exposure to sunlight expressed in per cent.

Time of taking sample	Covered					Exposed				
	Depth in cm.				Average of rows	Depth in cm.				Average of rows
9:00 A.M.	105	105	100	90	100	109	108	89	94	100
1:00 P.M.	88	119	88	109	101	66	77	83	89	79
4:00 P.M.	81	78	96	91	88	55	82	80	80	74
9:00 P.M.	84	90	90	105	92	54	71	65	96	71
Average of columns	90	98	93	99		71	85	79	90	
S.D. of one observation	10.2	20.2	8.7	13.5		25.6	21.5	16.4	14.5	

or the bottom, but at the 20-cm. level the difference between 16.4 and 8.7 corresponds to a chance of 2 in 100.

The evidence points to some influence producing an unexpectedly high variability at the 10-cm. level in the covered jar. Adopting 14, corresponding to the 35-cm. level as the residual S.D. of a single determination, that of the difference between averages of three would be

$$\frac{\sqrt{2}}{\sqrt{3}} 14 = 11.41.$$

Accordingly, a difference of twice this, or 22.8, corresponds to the 5 per cent level and $2.6 \times 11.41 = 29.6$ corresponds to the 1 per cent level of significance, approximately neglecting corrections for sample size. To take into account the effect of sample size, Fisher's Table IV should be used.

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THE CHEMICAL COMPOSITION OF THE CRYSTALLINE STYLE AND OF THE GASTRIC SHIELD: WITH SOME NEW OBSERVATIONS ON THE OCCURRENCE OF THE STYLE OXIDASE

C. BERKELEY

(From the Pacific Biological Station, Nanaimo, B. C.)

The Crystalline Style

The chemical nature of the crystalline style in the lamellibranchs has interested observers from quite early times. Nelson (1918) gives an excellent summary of the opinions held on the subject up to the time of the publication of his paper and concludes that the style is " a structure of colloid nature resembling mucin."

The view that the constitution of the material of the style resembles that of mucin seems to have originated with Barrois (1889), who showed in the case of *Cardium* that it behaved like a globulin in respect of its solubility and chemical reactions, but also had the property of yielding a reducing substance on hydrolysis with dilute acid. In the absence of carbohydrate substances capable of yielding a sugar on hydrolysis he concluded that this indicated the presence of mucin or chondrin. This conclusion is quoted in some of the early papers in which the constitution of the style of other lamellibranchs is discussed (e.g., List, 1902, in the case of *Mytilus,* and Yonge, 1926, in that of *Ostrea*), but without presentation of any further evidence. More recently reference to mucin is omitted and the substance of the style is regarded as " a protein of a globulin nature " (Yonge, 1931 and 1932). Previously to undertaking the present work I had myself completely confirmed Barrois' observations in the case of a number of species, but his conclusion did not seem justified in the absence of more complete knowledge of the nature of the reducing substances resulting from the hydrolysis of the style material. Any glucoprotein would, for instance, react in the manner indicated and no case is made out for mucin or chondrin in particular.

A good deal of confusion existed as to the respective meanings of these two terms until the matter was clarified by the work of Levene and his co-workers (1922). While formerly substances were classified as chondrins or mucins largely on the basis of their physiological origin and physical condition, provided they had the chemical properties of

proteins coupled with that of yielding a reducing substance on acid hydrolysis, the terms have now a definite chemical significance. In both cases the protein radicle is combined with a complex acid radicle, in that of the chondrins chondroitin sulphuric acid, in that of the mucins mucoitin sulphuric acid. Both of these acids yield on acid hydrolysis glucuronic acid, sulphuric acid, and an acetylated hexosamine, in the former case acetyl-galactosamine, in the latter acetyl-glucosamine. The presence of either mucin or chondrin can, accordingly, be definitely established by the detection of these substances among the products of hydrolysis.

The styles of the following four species of lamellibranch have been examined from this point of view: *Schizothærus nuttalli, Mya arenaria, Ostrea gigas,* and *Saxidomus giganteus.* These four species were selected partly because of their large size and ready availability in sufficient quantity to supply enough material for analysis, and partly because they exemplify two distinct types of crystalline style. Those of the two first-named species are very solid in texture, dissolve only slowly in distilled water, and remain practically intact when the animals are kept under adverse conditions even until death ensues. Those of the two last-named species, on the contrary, are of a much less solid texture, dissolve readily in distilled water, and disappear very rapidly when the animals are kept out of water. In the cases of all three species of "clam" the styles were removed immediately after the animals were dug, dried in a water-oven to constant weight, and stored for analysis. In that of the oyster this procedure could not be followed exactly because it was so frequently found that the style had already begun to soften, or had entirely disappeared, in animals left exposed on the beds by the receding tide. In this case, therefore, the animals were lifted into a large floating perforated tray and left there for some hours until the styles were found fully developed. These were removed immediately after the oyster was taken from the water and stored in alcohol until they could be transferred to the drying oven.

Glucuronic Acid

Glucuronic acid is readily detected in solutions containing relatively little other organic matter by its property of yielding furfurol on boiling with hydrochloric acid. In the presence of any of several polyvalent phenolic substances furfurol gives a series of highly colored compounds. To the solution under test an equal volume of concentrated hydrochloric acid is added and a trace of phloroglucin or orcin; on boiling a violet-red color develops with the former reagent, a violet-blue with the latter.

In application to crystalline style material this test gave inconclusive results since charring of the protein darkened the solutions too much for color-reading to be possible. As used quantitatively, this difficulty is overcome by distilling off the furfurol produced, which can be readily recognised in the distillate by allowing a drop to fall upon aniline-acetate test paper with which it gives a bright cherry red coloration. By this means positive results were obtained from the crystalline styles of each of the four species under consideration. A quantitative comparison was therefore made between them by continuing the distillation in each case until no more furfurol was produced, precipitating it from the distillate with phloroglucin, and weighing as phloroglucide. The detailed procedure was that of the method of Tollens and Kröber for the determination of furfurol derived from pentoses and the results in Table I are calculated from the phloroglucide by Kröber's factor (Browne, 1912).

TABLE I

Species	Weight of Material	Furfurol	Furfurol
			per cent
Schizothaerus nutalli	.5758	.007083	1.23
Mya arenaria	.5939	.007134	1.20
Ostrea gigas	.3890	.004343	1.11
Saxidomus giganteus	.4784	.004653	.97

The four species thus approximate to one another in furfurol-yielding capacity, and hence in the glucuronic acid content, of their crystalline styles, but such difference as occurs indicates a consistently lower content in the more soluble styles.

GLUCOSAMINE

The method described by Elson and Morgan (1933) for the determination of glucosamine and chondrosamine was applied qualitatively to all the acid residues from which the furfurol had been distilled in the determinations of glucuronic acid described in the previous paragraph. If mucin or chondrin were present in the crystalline style, it was anticipated that the corresponding amino sugar would remain in the solutions as hydrochloride after boiling with hydrochloric acid. For this reason the method of Elson and Morgan for glucosamine and chondrosamine was employed rather than the modification for the estimation of the acetylated compounds described in their later paper (1934). The solutions were made up to equal volume and 5 cc. of each was neutralised, filtered, and submitted to the test. In each case a positive reaction was

obtained while a blank test with the reagents remained entirely colorless.

A comparison was made quantitatively between the style of *Ostrea gigas* and that of *Schizothærus nutalli*. The residual solutions from the furfurol distillations were again used for this purpose. It was necessary in the first place to ensure that all the amine present had been set free from combination. To this end 5 cc. of each of the solutions was evaporated to small volume and the residue taken up with 5 cc. concentrated HCl and heated in a boiling water bath for two hours under a reflux condenser. The resulting solutions were neutralised and filtered, whereby only slightly colored filtrates were obtained, and the depth of color developed in Elson and Morgan's test compared with that from 5 cc. of the original solution after neutralisation and filtration. All the filtrates were made up to like volume before applying the test. In neither case could any alteration in the depth of color be detected as a result of the further heating with HCl. It was concluded, therefore, that the hydrolysis had already reached a maximum in respect of the amine constituent in the residues from the furfurol determinations. Comparison was accordingly made between the solutions without further hydrolysis.

The weights of *Ostrea* and *Schizothærus* material respectively taken for the furfurol determinations were almost exactly in the ratio of 1:1.5 (see table), so that volumes of the residual solutions taken in this ratio represented equal weights of material. Accordingly 5 cc. of the solution obtained from *Schizothærus* and 7.5 of that from *Ostrea* were withdrawn, neutralised, filtered, the filtrates made up to equal volume, and tested. Very little difference could be detected in the colors developed on standing, indicating a practical equality in the glucosamine content in the styles of the two species.

Sulphuric and Acetic Acids

A series of experiments parallel to those described in the foregoing paragraph was carried out with the residues from the furfurol distillations for the detection and attempted estimation of sulphuric acid, substituting precipitation with barium chloride in acid solution for the Elson and Morgan test.

All the solutions gave positive reactions, but the amounts of precipitate obtained from 5-cc. samples were very small and, in the case of *Ostrea*, only just detectable. In 25-cc. samples the precipitates were still too small to determine gravimetrically and further hydrolysis led to no perceptible increase in the amounts. Attempts at quantitative determination were therefore abandoned, but rough estimates were made

by eye of the relative amounts of precipitate obtained from aliquots of the four solutions representing equal weights of material. These pointed fairly definitely to the presence of more sulphuric acid in the styles of *Schizothærus* and *Mya* than in those of *Saxidomus* and *Ostrea*, the last named being markedly the poorest in that constituent.

No attempt was made to detect the acetic acid radicle in the residues from the furfurol determinations. There is no method by which the trace whose occurrence would be anticipated from the small amount of material taken for analysis could be recognised with certainty in the presence of the relatively large amount of hydrochloric acid.

Discussion

It has thus been shown that the crystalline styles of the four species investigated contain the essential constituents of the acids characteristic of mucin and chondrin. Elson and Morgan's test does not serve to differentiate glucosamine and chondrosamine, the respective amino sugars of these two acids, so that a definite conclusion cannot be drawn as to which of them is present in the style. A great deal more material than could readily be obtained would be necessary for this purpose, but, judging by its solubility and ease of hydrolysis with acid, it is extremely probable that mucin rather than chondrin is involved.

There seem to be some slight differences in the composition of the styles of the four species investigated, those of *Schizothærus* and *Ostrea*, for instance, agreeing very closely in their content of glucosamine, but differing in that of glucuronic and sulphuric acids. This suggests that the material consists of a mixture of mucin and a glucoprotein having glucosamine as its carbohydrate constituent; or, in other words, of a glucosamine glucoprotein a variable part of which is united with mucoitin sulphuric acid to form mucin.

If this be so, the differences in solubility of the styles in various species may be associated with their content of mucin. Yonge (1932) has shown that the readiness with which the style disappears when the living animals are exposed to adverse conditions can be correlated with the presence or absence of a separate style-sac; those species in which this is not present losing their styles more readily, when the secretion of the style material is inhibited, owing to their exposure to the solvent action of the digestive fluid of the stomach and intestine. While this is undoubtedly the case, it does not seem to explain the variation in solubility of the styles of various species when removed from the animal and placed in distilled water. *Schizothærus* affords an instance of an animal with a complete style-sac, *Ostrea* of one with none. In the former case, the style persists after long periods of adverse conditions, in the latter

it disappears very rapidly; but, as has been pointed out, the style of *Schizothærus* dissolves very slowly in distilled water and that of *Ostrea* very quickly and the former appears to have a greater amount of its glucoprotein in mucin combination. Chemical composition, therefore, may also be a factor in the solubility of the style material and influence its rate of disappearance in adverse circumstances under the action of the digestive fluid.

The Gastric Shield

The only statement I have been able to trace in connection with the chemical composition of the gastric shield is that of Nelson (1918). "From its consistency and action toward common reagents" this author concludes "it is probably in the nature of chondrin." This conclusion is quoted by Yonge (1926) in support of his view that "the gastric shield is not a secretion, but is formed by the fusion of cilia, originally in response to the irritation caused by the head of the style."

Schizothærus nutalli has a large gastric shield readily separable from the stomach wall and it was obtainable in fairly large quantity. It was therefore taken for investigation.

It was found at an early stage that the shield could be boiled with 60 per cent potash without dissolving or undergoing appreciable decomposition, the structure preserving its characteristic shape even after prolonged boiling and standing. This rendered a chondrin-like composition extremely doubtful since all chondroid substances hitherto investigated have been found to hydrolyse on alkaline digestion. Chitin seemed to be the compound having the property of resisting attack by strong alkali most likely to occur in the situation in which the gastric shield is found and the material was accordingly examined from this standpoint. After boiling for some time with 60 per cent potash, it was thoroughly washed and dried. It was then found to give the reaction characteristic of chitin with sulphuric acid and iodine and to dissolve readily in boiling concentrated hydrochloric acid. The solution in hydrochloric acid gave off ammonia on making alkaline and boiling, it had a strong reducing action on Fehling's solution, and gave a crystalline osazone with phenylhydrazine. All these tests pointed to chitin. Finally some of the material was submitted to the treatment described in the earlier part of this paper for identifying the constituents of the crystalline style. On boiling with hydrochloric acid and distilling, no furfurol could be detected in the distillate. The residual solution gave no reaction for sulphuric acid, but the presence of glucosamine was shown by the test of Elson and Morgan. Chitin consists of polymerised acetyl glucosamine. There is thus no doubt that the substance

of which the gastric shield is composed is chitin and that it contains no chondrin-like constituent.

Nelson's (1918) observation that the gastric shield gives the biuret test and breaks up in the process under the action of the strong alkali employed is probably to be attributed to an incomplete separation of the adjacent tissues and of the substance of the crystalline style which is always more or less adherent to it.

As bearing on Yonge's (1926) view of the origin of the gastric shield, it is of interest to speculate whether the shield may not be derived from the substance of the style itself. From the chemical standpoint it does not seem impossible that chitin could be formed from mucin by the elimination of glucuronic and sulphuric acids from mucoitin-sulphuric acid and polymerisation of the remaining acetyl glucosamine. The related compositions of mucin and chitin have previously been discussed from this point of view by Matthews (1916), who draws attention to the occurrence in large quantity of mucin in the skin of the lower vertebrates and of chitin in the hard covering of the arthropods and considers that the " facts are of interest in the light of the theory of Gaskell and Patten that the arthropods were the ancestors of the vertebrates."

The Style Oxidase

The occurrence of an oxidase system in the crystalline style of the lamellibranchs has hitherto been recorded in the cases of thirteen species which I have enumerated in a previous paper (Berkeley, 1933). Two more, *Panope genorosa* and *Pholadidea penita,* may now be added to the list. I have recently detected considerable oxidase activity in solutions of the styles of both of these. The oxidase has now been observed in a sufficiently large range of families to render it extremely probable that its occurrence is general throughout the lamellibranchs.

The presence of the oxidase has not, hitherto, been recorded in the style of any species of gastropod. Unlike the case of the lamellibranchs, the style is by no means of general occurrence in the gastropods. Its presence has, in fact, been observed in only a very limited number of genera. Yonge (1932) gives a complete list of these and points out that the style occurs only in such gastropods as are herbivores and which " either by ciliary currents or by a radula, pass a continuous supply of finely divided food to the stomach."

The only large marine species among them which I have been able to obtain in a living condition is *Crepidula fornicata.* This species is common in the oyster beds at Olympia, Washington, whence a number of live specimens were recently obtained. On arrival, some 24 hours after being taken from the beds, none of those opened had any crystal-

line styles. After being kept in the sea for some days, a large number died, but all the survivors contained styles and supplied sufficient material to enable me to carry out a series of oxidase tests. The results of these were quite definitely positive.

Summary

The crystalline styles of four species of lamellibranch have been examined chemically to determine whether the material is entirely protein in nature or contains mucin or chondrin.

The material yields on acid hydrolysis, glucuronic acid, sulphuric acid and a hexosamine, in addition to protein. It therefore contains all the essential constituents of mucin or chondrin. The solubility and ease of hydrolysis of the material suggest that mucin rather than chondrin is involved.

The composition of the styles is not quantitatively identical in the four species examined, but varies in such a way as to suggest that the less readily soluble styles contain the larger quantity of mucin.

The material of which the gastric shield is composed is found to be chitin.

The oxidase system previously recorded in the styles of a number of lamellibranchs is now shown to occur in two more species. It is also found to be present in that of the gastropod, *Crepidula fornicata*.

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A QUANTITATIVE STUDY OF THE VERTICAL DISTRIBUTION OF THE LARGER ZOÖPLANKTON IN DEEP WATER

BENJAMIN B. LEAVITT

(From the Woods Hole Oceanographic Institution,[1] Woods Hole, Massachusetts)

It is now generally accepted that all depths of the ocean are inhabited (Murray and Hjort, 1912, Chap. IX), but the variations in the amount of plankton at different depths have not yet been investigated thoroughly. The data herein presented are meagre but so little actually pertinent data have been gathered previously that they are more valuable than their paucity might suggest.

During the summer of 1933, investigation was undertaken of the quantitative vertical distribution of pelagic animals in deep water in the offing of Woods Hole. It is believed that the nets used (stramin, 6 threads per centimeter) are as adequate as any gear yet devised for sampling the invertebrate communities as a whole, including the bathypelagic fishes up to a size of six inches or so in length. Obviously the giant squids and active large fishes are not proper quarry for tow nets. The micro-zoöplankton is not caught in nets of such coarse mesh; very different methods must be applied to the investigation of microscopic forms.

It has been possible to measure the total volumes of all the catches and to identify and count all the Euphausiacea. It is hoped that in the near future the species of other groups of animals may be identified and the various problems of distribution concerning them may be attacked.

Work at sea was carried on aboard the research vessel "Atlantis." The collections were made 100 to 300 miles south and east of Woods Hole. The locations of the stations were as follows: No. 1733 (36° 54′ N. and 68° 15′ W. to 36° 35′ N. and 68° 33′ W.) July 27–28, 1933; 2500 fathoms. No. 1735 (36° 50′ N. and 69° 16′ W. to 36° 50′ N. and 68° 52′ W.) July 28–29, 1933; 2500 fathoms. No. 1737 (39° 29′ N. and 70° 14′ W. to 39° 46′ N. and 70° 09′ W.) July 30–31, 1933; 1200 fathoms. No. 1739 (39° 42′ N. and 67° 04′ W.) August 12–18, 1933; 2009 fathoms. Of these four stations, one (1733) was located in the offshore edge of the Gulf Stream; one (1735) in the axis of the latter; and one (1737) in slope water inshore from it. The August

[1] Contribution No. 59.

station (1739) was just outside the continental slope well inshore from the edge of the stream.

Because of the danger of contamination always present when open nets are used, it has long been appreciated that for the collection of the larger planktonic animals from a known depth nets are needed that can be lowered closed to the desired depths, then opened, towed horizontally the desired period of time, then again closed before being hoisted. It is also desirable to use several nets simultaneously in series on the wire in order to save time and to eliminate changes of location or speed, and other variable conditions. Many attempts have been made to perfect gear of this sort. Chun (1888, 1903), Agassiz (1888), Fowler (1898), Kofoid (1905), Bigelow (1913), Ostenfeld and Jespersen (1924), Kemp, Hardy, and Mackintosh (1929), Harvey (1934), and others have devised and used closing nets of one kind or another. But, for technical reasons, none have yet been generally used, even on major deep sea expeditions; and for that reason, many of the published statements as to the vertical distribution of bathypelagic animals have rested on meagre evidence.

Kofoid (1911) gives a very good resumé of the various causes for the failure of most of the earlier attempts with closing nets. Some of the nets were too complex and delicate for the rough work at sea; some were too expensive for routine use; some were designed to be lowered open; some were to hang from the end of the wire and so could not be used in series; some were useful only for vertical tows, not for horizontal; and some were too small for the capture of the more active forms. Kofoid's own net was considered too small and too expensive for the present investigations.

To send down open tow nets to different depths and, by the differentiation of their contents, to assume that the differences in the catches reflect the differences in bathymetrical ranges of the species obtained is of practical application only in reasonably shallow tows. This method not only involves the necessity of a great many hauls in order to formulate significant conclusions, which in itself is enough to make it impractical in deep water, but the tremendous increases in contamination of deep tows by animals from the upper levels render this method completely valueless there.

The nets and releasing device described in Nansen (1915) would not serve our purposes as they are designed to be lowered open and as only one net can be used on the wire at one time. The nets and releasing device described by Ostenfeld and Jespersen (1924) are modeled after these and, whereas improvements have been made, the entire apparatus is designed for other purposes and is not applicable to the

investigation of the quantitative distribution of bathypelagic plankton in the open sea.

The " Discovery " investigators have tried various kinds of plankton nets and have also done some experimentation with releasing devices. The closing mechanism employed with their large nets is elaborate and expensive, and, as they point out (Kemp, Hardy, and Mackintosh, 1929, p. 196), " the results obtained with these large horizontal nets were not always satisfactory."

The releasing and towing mechanism used by Harvey (1934) is not of adequately rugged construction and the principles on which it works prohibit its use with the large strains involved with two-meter nets.

A closing net to be adapted for the collection of zoöplankton at great depths should meet the following requirements: (*a*) suitable for horizontal tows in order that one particular depth may be investigated and that a fair quantity from this depth be obtained; (*b*) suitable for use in series so that data on different depths may be gathered contemporaneously; (*c*) easy to handle; (*d*) inexpensive; (*e*) large enough to catch an appreciable amount of material at depths where life is not abundant and to catch large and active forms such as Euphausiids, decapods, etc.; (*f*) strong; (*g*) adapted to tow at one level, i.e., that at which it is opened.

The development of suitable gear required considerable experiment. It was thought at the outset that one-meter closing nets of the type described by Bigelow (1913) would be satisfactory, and these were used at Stations 1733, 1735, and 1737. While the releasing device (since improved) had a few minor weaknesses which caused some of the hauls to fail, on the whole this net worked well, and there is no reason to believe that most of the hauls made with it are not dependable samples of the faunas living at the depths where obtained. Mechanical difficulties, however, limit the use of this net to small sizes, and the catches obtained with it were so small as to point to the need of larger nets.

A system was therefore devised for the use of a two-meter net, opened and closed by a single draw-string (Fig. 1, *b, a, c*), operating through the mouth from the inside.

When lowered, the net is hung from the primary release (t_1) on the releasing device by an eye (*a*) spliced into the draw-line. The draw-line (*b a c*) is attached to a ring of rope (*c g c*) which passes through two eyes in the canvas belly band surrounding the net and thence through a series of rings fastened on the outside of the latter. While the net is being raised or lowered closed, the front part of the net itself is everted through the mouth to form a cone (Fig. 1, *A* and *C*). When the primary release (t_1) is tripped by the first messenger, the eye (*a*) is

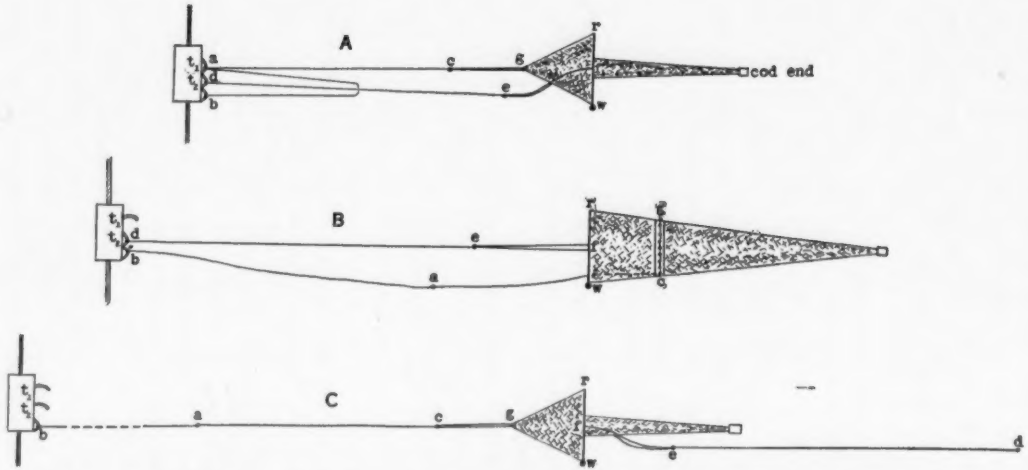

FIG. 1. Closing net used. *A*, net closed for lowering. *B*, net open for towing. *C*, net closed for raising. *b a c*, 9.7 and 6.7-meter draw-string. *c g c*, 7-meter purse line. *d e*, 9.5-meter towing line. *e f*, 3.2-meter towing bridle. *r*, 2-meter diameter rim. *w*, 37-lb. weight. t_1, primary release. t_2, secondary release.

cast off and the force of the water, pouring against the mouth of the net as the ship moves ahead, forces the net open, the pursing line slipping freely through the rings on the outside of the belly band. The pull of the net then comes onto the towing line (*d e*) connected with the bridles (*e f*) (Fig. 1, *B*). While towing, the draw-line (*b a c*) is so slack that the net opens out and operates. After the net has towed open for the allotted time, a second messenger is dispatched which trips the secondary release (t_2), the towing line is thus cast off, and the net comes again onto the draw-line (*b a c*), the end of which is permanently fast to the releasing device. The pull on the draw-line then operates the pursing line around the middle of the net and it is thus closed (Fig. 1, *C*).

When tows with two-meter nets are made from the "Atlantis," it is found most practical to use the ½" cable with an eight hundred pound weight on the end, to aid in keeping a low wire angle.

After some experiments with the gear, we succeeded in making twenty-nine deep hauls which may be dealt with from a quantitative point of view.

The tows at Station 1739 were all made in an oblique manner through a given stratum of water, by hauling in a prescribed number of meters of wire every five minutes in such a way that the duration of the tow was approximately two hours in most cases. At the other three stations, all tows were horizontal. The best speed at which to tow, keep a reasonably small wire angle, and have the net strain the water well, is about one and one half to two miles an hour. This speed was maintained in so far as possible for all tows, which in turn means that the corrected quantity of plankton caught is roughly that contained in a column of water two meters in diameter and four miles long.

The exact geographic position where each tow began and ended is not recorded here, since it may be assumed that hydrologic conditions were reasonably constant within the region designated by the one determination of latitude and longitude given above for each station. Care was taken not to run far from the given positions, and tows were made in different directions, thus confining them all to an area not too extensive.

The catch from each tow, after the removal of any unusual, large, or delicate specimens, was anaesthetized by the addition of a saturated solution of chloretone and subsequently preserved in dilute formalin. Anaesthetizing plankton before preservation has proven wise in view of the fact that many planktonic animals, particularly the Crustacea, are endowed with the powers of autotomy, and so if placed directly in an

irritating preservative, may cast off parts which are of importance in species determination.

In ascertaining the volume of plankton in a given tow, the entire catch is placed on a silk bolting cloth strainer and allowed to drain, but not to dry. It is then added to a known quantity of preservative in a graduated cylinder and the displacement volume of the animals is determined by subtracting the known from the total.

The preliminary sorting and counting were done by the methods used by Mackintosh in dealing with the macro-zoöplankton in the Atlantic sector of the Antarctic (Mackintosh, 1934, pp. 70–72).

As differences in size of mesh of the nets, in speed of towing through the water etc. all cause differences in the amount of plankton caught, it is obvious that uniformity of procedure is essential if the results of different hauls are to be directly comparable one with another. The preliminary hauls here discussed, in which it was necessary to experiment with various nets and with various towing speeds, obviously do not meet the above standard. Therefore, to express the relative abundance I have corrected all tows to the calculated amount that would theoretically have been taken by a net two meters in diameter in two hours towing (Table I, column 8). Even after correction, however, there are still so many unknown factors that the result can only be regarded as a rough estimate of the relative quantity of animals that were living at the time and place where they were caught.

In Table I, the volume of plankton caught is given as measured by displacement, after the salpae have been removed. The removal of the salpae is necessary because their physical properties are such that they do not lend themselves to measurement by the displacement method or any other yet devised. These animals are, however, an important factor in the plankton community and in the metabolism of the sea because at certain times and places there is more material in the form of salpae than in all other types of plankton combined.

The diameter of the net used refers to the diameter across the mouth. The smaller net was scrim, the one-and-one-half-meter net was of silk, and the two-meter nets were of stramin.

Comparison of the quantities of plankton caught at different depths, both by volumes and on a percentage basis, shows clearly that the largest amounts were caught in the upper 300 meters.

At Station 1739 over 93 per cent of the plankton caught was taken between 800 meters and the surface in four out of thirteen hauls. If we subtract the volume of salpae which constituted over 80 per cent of the total catch, approximately 74 per cent was taken between 800 meters

TABLE I

Station no.	Haul no.	Diam. of net used	Duration of tow	Depth	Percentage of total catch at station including salpae	Volume of plankton caught minus salpae	Calc. volume plankton per 2-m. net in 2 hrs. minus salpae
		m.	*hr.*	*m.*	*per cent*	*cc.*	*cc.*
1733	1	1	1	25	55	24	192
"	2	1	1	100	12	5	40
"	3	1	1	300	8	3.5	28
"	5	1	1	300	12	5	40
"	4	1	1	700	13	6	48
					100		
1735	1	1	1	25	29	25	200
"	2	1	1	100	23	20	160
"	3	1	1	300	7	6	48
"	4	1	1	700	8	7	56
"	5	1	2	1200	4	4	16
"	6	1	2	2000	29	25	100
					100		
1737	5	1	1	25	44	120	960
"	4	1	1	100	21	40	320
"	3	1	1	300	28	35	280
"	2	1	1	700	3	35	280
"	6	1	2	1200	.3	5	20
"	1	1	2	2000	3	40	160
					99.3		
1739	1	2	2	400– 0	36	256	256
"	21	2	2	700– 0	14	326	326
"	21	2	2	700– 0	13	146	146
"	19	2	2	800– 500	31	356	356
"	16	2	2	1400–1000	1	18	18
"	17	2	2	1400–1000	1	40	40
"	17	1.5	2	1400–1000	1	6	10.7
"	15	2	2	2000–1600	1	43	43
"	15	2	2	2000–1600	1	57	57
"	13	1.5	1.33	2000–1600	1	15	32.4
"	3	1.5	2	2600–2200	1	85	151.3
"	4	1.5	1.33	3200–2800	2	120	256.3
					103		

and the surface and 26 per cent was taken in depths greater than 800 meters.

The percentages of quantities, including salpae, caught at different depths, at the three deepest stations are as follows:

Station 1735—58 per cent taken above 700 m., deepest haul at 2000 m.
" 1737—93 " " " " 700 " " " " 2000 "
" 1739—94 " " " " 800 " " " " 3200 "

It is true that vertical migrations force us to regard the population of the whole bathymetric province as peculiarly dynamic, particularly so in the photic zone. Nevertheless, it seems sufficiently established that on this particular occasion and at these localities from 75 per cent to over 90 per cent of the animals (by volume) were living in depths less than 800 meters. The works of Chun, Haeckel, Hensen, Fowler,

TABLE II

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1733.

	25 m.	100 m.	300 m.	300 m.	700 m.
Thysanopoda tricuspidata	8				
	2.08				
Thysanopoda orientalis		16			
		20			
Euphausia tenera			105		
			840		
Euphausia hemigibba	40		9		
	10.40		*72*		
Euphausia mutica	496				
	128.96				
Thysanoessa gregaria			5	24	38
			40	*43.2*	*304*
Thysanoessa inermis			2		
			16		
Nematoscelis megalops		32			1
		40			*8*
Nematoscelis atlantica		72			
		90			
Nematoscelis tenella				8	
				14.4	
Stylocheiron carinatum	1824				
	474.24				
Stylocheiron longicorne		248	27	48	1
		310	*216*	*86.4*	*8*
Stylocheiron elongatum		8	21	64	
		10	*168*	*115.2*	
Nematoscelis sp.		8			
		10			
Young euphausids		48			
		60			

and many others (Steuer 1910, Kapitel V) point to the fact that it is not surprising to find the proportion of animals in the upper eight hundred meters as large as 90 per cent.

The data also show that there was a minimum quantity of plankton at a depth of 1200 meters, with the quantities increasing progressively both toward greater and toward lesser depths.

This barren stratum is probably what Agassiz (1888) regarded as an azoic zone, and while not actually as poor in animal life as he supposed, the contrast between it and the waters above was striking enough. The explanation of this peculiar distribution is not clear, and whether or not it is a permanent condition fairly universal in its extent in the open sea is an open question, the variation in the abundance even of individual species being often great, both within very short distances, and within very short intervals of time. For example, Michael's (1911) statistics on the numbers of chaetognaths show great differences with successive hauls, both at the same place and in closely proximate places. Single hauls at separate localities can only be regarded as proof that what was caught was present at the particular time and place where the tow was made.

The increase in abundance of plankton in the deep water below the barren zone centering around 1200 meters was thought by Agassiz (1888) to be due to animals gleaning their living from the bottom itself, or to larvae of the bottom fauna which are pelagic during a part of their life histories. This was not the case in the present instance, for at Station 1739, the animals collected at a depth of 3,200 meters (some 600 or 800 meters from the bottom) were a truly pelagic community, i.e. in no way directly dependent on the bottom itself. Thus copepods were abundant throughout the depths, a species of *Metridia* being exceedingly numerous in one haul at a depth of 2,000 meters. Other groups of pelagic animals such as acanthephyridae and other decapods, pelagic ostracods, siphonophores, euphausiids, fish, etc. were represented at all depths from surface down into deep water. Until the species of other groups of animals from these collections have been examined in detail, it would be premature to state just which ones were responsible for the increase in volume of plankton at depths greater than 1,200 meters.

Numbers of Euphausidae

Besides studying the absolute abundance of the total plankton at various depths, it is interesting to learn the relative numerical abundance, one to another, of the individual species in the plankton community. This will clarify two problems concerning the animals caught. First, the relative numbers of animals of the various species at different depths. Second, the number present per unit volume of catch, which tells the proportionate importance of a species or group as compared with the rest of the animals present, i.e. the quantitative rôle which they play as a part of the animal community. In studies of this sort, different investigators have often used such terms as "dominant," "many," "few," "rare," "present," etc. to express quantities; but all such ex-

TABLE III

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1735.

	25 m.	100 m.	300 m.	700 m.	1200 m.	2000 m.
Thysanopoda orientalis			16			
			16			
" *pectinata*			8			
			8			
" *acutifrons*						4
						2
" *aequalis*		8				
		2.48				
Euphausia tenera	992	112		16		
	248	*34.72*		*14.08*		
" *americana*	128	8				
	32	*2.48*				
" *hemigibba*		152				
		47.12				
" *gibboides*		32				
		9.92				
" *brevis*	384					
	96					
Thysanoessa gregaria						432
						216
Nematoscelis atlantica				24		
				21.12		
" *tenella*		24	40			
		7.44	*40*			
" *microps*		104	48	72		
		32.24	*48*	*63.36*		
Nematobrachion flexipes			8			
			8			
" *sexspinosus*				8		
				7.04		
Stylocheiron longicorne			224	8		
			224	*7.04*		
" *maximum*				8		
				7.04		
" *insulare*		144				
		44.64				
" *suhmii*		1064				
		329.84				
" *carinatum*	9472	96				
	2368	*29.76*				
" *elongatum*	24		24			
	6		*24*			
" *affine*	8					
	2					
Euphausia sp.				8	28	
				7.04	*86.8*	
Young euphausids	2568	728		16		
	642	*225.68*		*14.08*		

pressions break down in practical use. "Dominant," for instance, is impractical because of the deceptions due to size, color, aggregations, etc., of various kinds of animals. Thus a few *Acanthephyras* may appear to dominate a tow or a single chain of *Salpae* may change the "complexion" of another. We are, therefore, reduced to the necessity of making actual counts for the determination of numbers.

The Euphausiacea are the only group of which total counts have as yet been made. In cases where one or two species which it is impossible to separate from others with the naked eye occur together, it has been found sufficient, for specific enumeration within the group, to separate out three samples of twenty specimens each, from a dish in

TABLE IV

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1737.

	25 m.	100 m.	300 m.	700 m.	1200 m.	2000 m.
Euphausia krohnii	16	152				
	.8	*22.80*				
" *gibboides*		8				
		1.20				
" *tenera*			8			
			1.44			
" sp.		8				
		1.20				
Thysanoessa gregaria	136	1544				
	6.80	*231.60*				
" *longicaudata*			104	1000	148	948
			18.72	*180*	*370*	*293.88*
Nematoscelis megalops	120	5224	3424			
	6	*783.60*	*616.32*			

which the whole catch of euphausiids has been isolated from the rest of the catch of plankton. They were first well stirred and then sampled by taking out the twenty animals lying nearest to a point on the edge of the dish. Identification under the microscope of three such samples of Euphausidae, from Station 1739, revealed *Euphausia americana, E. mutica,* and *E. brevis* in the following proportions:

Euphausia americana	16	15	18
" *mutica*	3	3	2
" *brevis*	1	2	0

As these counts agree so closely, it seemed justifiable to consider this sampling method adequate.

TABLE V

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1739.

	400–0 m.	700–0 m.	700–0 m.	800–500 m.	1400–1000 m.	1400–1000 m.	1400–1000 m.	2000–1600 m.	2000–1600 m.	2000–1600 m.	2600–2200 m.	3200–2800 m.
Thysanopoda aequalis		27										
		4.05										
" *orientalis*	1											
	.19											
" *tricuspidata*			1									
			.34									
" *acutifrons*				1								
				.14								
" sp.	2		7									
	.38		*2.38*									
Euphausia tenera	643	504	604									
	57.87	*75.60*	*205.36*									
" *hemigibba*	12	148	36									
	2.28	*22.2*	*12.24*									
" *pseudogibba*	5											
	.95											
" *krohnii*	462	375	106									
	79.78	*56.25*	*36.04*									
" *americana*	459	70	68									
	87.21	*10.50*	*23.12*									
" *brevis*	25											
	4.75											
" *mutica*	137											
	26.03											
" sp.	2			4								
	.38			*.56*								
Nematoscelis megalops	164	22	11	6								
	31.16	*3.30*	*3.74*	*.84*								
" *microps*	84	21	20	6								
	15.96	*3.15*	*6.80*	*.84*								

TABLE V—*Continued*

	400–0 m.	700–0 m.	700–0 m.	800–500 m.	1400–1000 m.	1400–1000 m.	1400–1000 m.	2000–1600 m.	2000–1600 m.	2000–1600 m.	2600–2200 m.	3200–2800 m.
Nematoscelis tenella		7	6									
		1.05	*2.04*									
Thysanoessa gregaria	12							1	2			
	2.28							*1.16*	*1.76*			
" *parva*				45		84		20	2	277.55	36	401.4
				6.3		*105*		*23.20*	*1.76*	*416.32*	*11.88*	*76.26*
" *longicaudata*		2	63	2		19						
		.3	*21.42*	*.28*		*23.75*						
" sp.		114			7							
		17.10			*19.6*							
Stylocherion maximum	238											
	45.22											
" *carinatum*		1	3									
		.15	*1.02*									
" *affine*			3									
			1.02									
" *abbreviatum*		15										
		2.25										
" *elongatum*		7	7									
		1.05	*2.38*									
" *longicorne*	1											
	.19											
" sp.		6										
		.90										
Nematobrachion boopis		1										
		.15										
Bentheuphausia ambylops						5	1.78	1	1			
						6.25	*8.07*	*1.16*	*.88*			
Young euphausids			9									
			3.06									

The number of each species of euphausiids has been obtained either by actual count or by the above-mentioned sampling method. To make all catches comparable, the counts of each individual species have then been reduced to a 2-meter net in 2 hours basis. A simple additional calculation of numbers of euphausiids per unit volume (50 cc. of total catch) then shows the relative importance of the former in the latter.

The following example will illustrate this method of calculation; Station 1735; Haul 2; depth 100 m.; total catch 20 cc.; net used 1 m.;

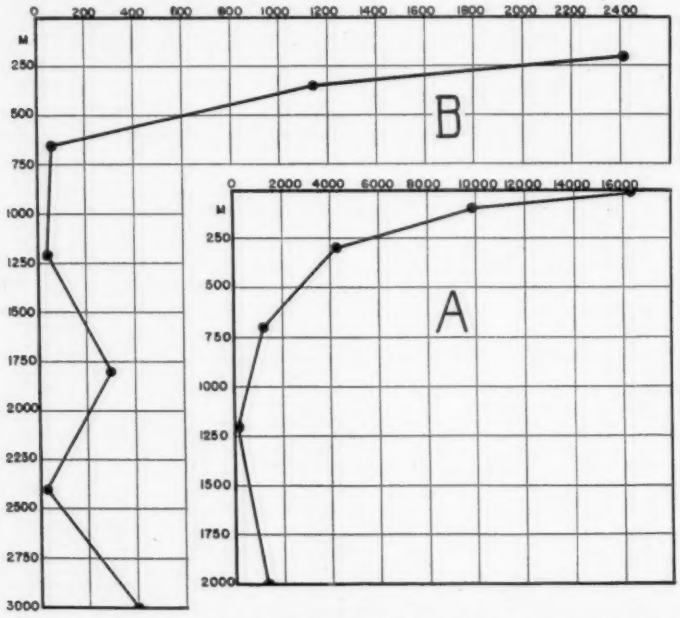

FIG. 2. *A*, Total numbers of euphausiids calculated per 2-meter net per 2-hour haul, Stations 1733, 1735, and 1737. *B*, Total numbers of euphausiids, per 2-meter net per 2-hour haul, Station 1739, determined from average catch at average depths.

duration of tow 1 hr.; cc. per 2-meter net in 2 hrs. 160; number of *Euphausia tenera* actually present 14; number of *E. tenera* in 160 cc. 112; number of *E. tenera* in 50 cc., per 2-hour tow with a 2-meter net, 34.72.

Figure 2 shows that the number of euphausiids of all kinds decreased quite regularly with increasing depth at Stations 1733, 1735, and 1737, down to 1,200 meters, with no trace of a secondary maximum at about 700 meters such as appeared in the vertical distribution of plankton vol-

umes at the time. And while numbers of euphausiids like quantities of plankton increased below 1,200 meters, the importance of this group in the total animal community decreased at these same stations right down to the deepest layer sampled (Fig. 3, *A*), the number of euphausiids per 50 cc. of plankton being much smaller at depths greater than 500 meters than in the upper 300 meters. This vertical relationship

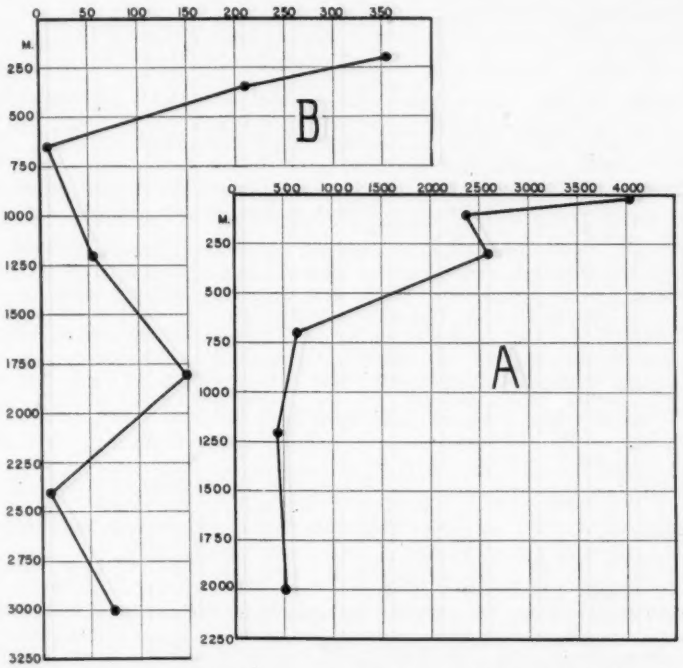

FIG. 3. *A*, Calculated numbers of euphausiids per 50 cc. of plankton from different depths at Stations 1733, 1735, and 1737. *B*, Calculated numbers of euphausiids per 50 cc. of plankton from different depths at Station 1739, determined from average catch at average depths.

also obtained for Station 1739 (Fig. 3, *B*), though at this locality there was a secondary maximum in relative importance of euphausiids at about 1,800 meters.

This decrease, with increasing depth, both in numbers and relative importance of euphausiids, is furthermore accompanied by a corresponding decrease in the specific diversity of the group, the counts listed in Tables II–V showing that whereas some fifteen species are taken in

the upper 400 meters of water, only five (*Thysanoessa longicaudata, T. gregaria, Bentheuphausia ambylops, Euphausia acutifrons,* and *Thysanoessa parva*) were found at depths greater than 800 meters. And of these five, the last two can alone be regarded as truly bathypelagic species on the basis of the present evidence.

Thus it seems sufficiently established at least for this part of the ocean and time of year that euphausiids as a group reach their highest development in the upper water layers.

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OBSERVATIONS ON THE COLOR CHANGES AND ISOLATED SCALE ERYTHROPHORES OF THE SQUIRREL FISH, HOLOCENTRUS ASCENSIONIS (OSBECK)

DIETRICH C. SMITH AND MARGARET T. SMITH

(From the Bermuda Biological Station and the University of Tennessee, Memphis, Tennessee)

On transference from a dark to a light-colored background the common Bermudian squirrel fish, *Holocentrus ascensionis* (Osbeck), shows an exceptionally rapid color change from red to white. This change is coincident with and the result of a pigment concentration within the numerous erythrophores of the scales and the deeper layers of the skin. The speed with which this is effected is strikingly demonstrated when a red fish from a dark background (in these experiments such backgrounds were usually black) is placed in a white aquarium containing three or four animals in the pale condition. In five seconds the originally red fish is indistinguishable from the earlier occupants. Similarly a pale fish placed in the company of red fishes in an aquarium over a black background loses its distinctive coloring in about ten seconds. Such rapid color changes are rarely met with among the fishes, at least under laboratory conditions, although they are not uncommon among the lizards. In the scorpion fish of the Bay of Naples, *Scorpæna ustulata*, a red form comparable in color to *Holocentrus* with erythrophores as the dominating type of pigment cell, the shift from red to the pale condition is a matter of hours (Smith and Smith, 1934). In melanophores the change in distribution of the pigment granules in response to a variation in background from white to black or vice versa is usually completed in three to four minutes. The melanophores of *Fundulus heteroclitus* behave typically in this manner.

The rapidity with which *Holocentrus* is capable of altering the distribution of pigment within its erythrophores is suggestive of a high degree of nervous control over the activities of the chromatophores. Such a supposition is borne out by experiments based upon the methods originally employed by Pouchet (1876), von Frisch (1911, 1912) and others in working out the relationship between the nervous system and chromatophores of fishes. These methods rely principally upon sectioning the sympathetic chain at various levels along its course and noting the effect of such operations upon the subsequent behavior of the pig-

ment cells in the area affected by the operation. In *Holocentrus* if the section involves the anterior half of the sympathetic chain, the area in front of the cut turns red and stays red regardless of background. If the cut, on the other hand, involves the posterior half of the chain the reddened area is confined to the regions behind the cut. In *Holocentrus* severance of the sympathetic then results in a typically persistent expansion of the denervated erythrophores, the distribution of the pigment-motor fibers to these erythrophores conforming to the scheme originally announced by von Frisch (1911) as applying to the innervation of the melanophores of *Phoxinus lævis*. In both species the pigment-motor fibers emerge from the spinal cord at a point about halfway down the trunk and are distributed both anteriorly and posteriorly by way of the sympathetics to the chromatophores in question. Operations of the nature just described demonstrate clearly the existence of sympathetic pigment-motor fibers capable of governing the responses of the erythrophores of *Holocentrus*.

In operated fishes kept upon a white background the resultant red or denervated areas produced by cutting the posterior sympathetic chain were visible in whole or in part for ten or fifteen days after the operation. In such denervated regions the first sign of recovery of function on the part of the affected erythrophores appeared in those cells lying most anteriorly in the operated area. In animals kept upon a white background this portion paled within three or four days after the operation, the paling gradually spreading each day more and more posteriorly until finally the whole area had lost its red color. Such animals when placed on a dark background showed a uniform reddening over the entire body. Repetition of the operation at its original site led to an exact duplication of the original reddening. This reëstablishment of the denervated condition following a second operation together with the nature of the recovery from the original operation is not in disagreement with the assumption that there is a gradual regrowth of nerves into the denervated region together with reëstablishment of nervous connections with the erythrophores. Denervations of the erythrophores of the anterior half of the trunk present a different picture. Here the recovery is much more rapid, so rapid in fact that the possibility of nervous regeneration is excluded. Subsequent operations at the original site often failed to produce the original results. In fact, the general impression received after comparing the behavior of denervated areas in the head and anterior trunk with those in the posterior trunk strongly suggest some essential difference between the innervation of the erythrophores, or else, what seems even less likely, a structural or functional difference between the erythrophores in these two parts of the body. Unfortu-

nately the opportunity was not given us to pursue this matter further. A somewhat similar relationship between dorsal and ventral trunk melanophores in *Phoxinus* has also been reported (Smith, 1931).

Further proof of the nervous control of the erythrophores in *Holocentrus* is obtainable on stimulating the anterior end of the medulla where, following the work of von Frisch (1911), one would expect to find the pigment-motor center. When by means of a suitable stimulating electrode this is done in *Holocentrus* the entire animal immediately pales and stays pale as long as the stimulus is applied. On stopping the stimulation the animal reddens, the erythrophore pigment assuming the state which typically follows the necessary operative procedure involved in exposure of the posterior portion of the brain. The original paling may be reproduced at will as long as the chromatophores remain in a reactive condition. Furthermore stimulation of the opthalmic nerve at

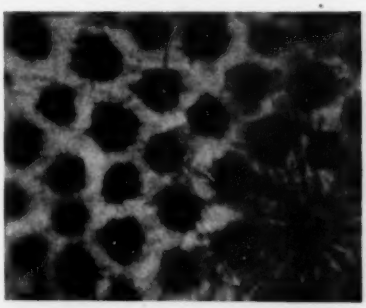

FIG. 1. Pulsating erythrophores in the expanded phase in N/10 NaCl. A non-pulsating expanded melanophore in the lower right-hand corner.

a point where it crosses the superior surface of the orbit produces a marked ipsilateral paling of the head (Pouchet, 1876; v. Frisch, 1911; Smith, 1931).

A reversal of the effect of temperature changes on the erythrophores of the trunk before and after sectioning the sympathetic chain gave further evidence of an innervation of these cells. Exposure of the unoperated trunk to a locally directed stream of warm sea water (35° C.) produced in the affected region a pronounced expansion of the erythrophores, as shown by the increase in the red color of the surface of the body at the point warmed. This reddening was strictly confined to the area heated. If, however, the stream were directed upon an area of the trunk in the same fish denervated a few days previously the erythrophores now contracted and the heated area became pale. A similarly directed stream of cold water produced in the innervated trunk a con-

traction and in the denervated trunk an expansion of the erythrophores. This curious reversal of the responses to heat and cold on the part of certain chromatophores after denervation has previously been noted in connection with the melanophores of *Fundulus heteroclitus* (Smith, 1928). Such reversals are not apparently the rule among fishes as attempts on other occasions to evoke the same response in the chromatophores of other forms (*Phoxinus lævis, Scorpæna ustulata, Trigla* sp.) have been unsuccessful, the direction of the response of the chromatophores of these fishes to temperature changes remaining the same whether denervated or innervated. This was also true with another Bermudian form, the surgeon fish, *Acanthurus hepatus*. The extent to which this peculiarity characteristic of the chromatophores of *Fundulus* and *Holocentrus* is spread among other fishes should be a matter worth investigating further.

FIG. 2. Pulsating erythrophores in the contracted phase in N/10 NaCl.

The scales of the squirrel fish upon microscopical examination proved to be favorable objects for direct observations upon the activity of the chromatophores. Three types were present, erythrophores, xanthophores, and melanophores, the erythrophores being the most numerous with an even distribution throughout the pigmented area of the scale, while the xanthophores, less abundant than the erythrophores, were largely confined to the distal corners. The melanophores were relatively scarce and were more or less irregularly scattered throughout the scale. In sea water isolated scales showed chromatophores in the semi-expanded condition, that is with only the proximal one-third or less of the processes of the cell filled with pigment. In N/10 NaCl, however, the pigment distribution was no longer a static affair, the erythrophores and the xanthophores instead alternating between states of expansion and contraction, the pigment granules migrating rhythmically in and

out of the processes (Figs. 1 and 2). These pulsations first appeared at the proximal border of the pigmented area, occurring almost simultaneously with the color changes of the iridocytes (Smith, 1933). This would indicate that the pulsations were an immediate response to the penetration of the NaCl into the tissues of the scale. The pulsations were relatively slow at first, beginning with two or three a minute, a level from which they gradually increased to between fifteen or twenty-five a minute. At this point the rate either gradually declined until pulsations ceased entirely or else it continued to increase further. In this latter event rates of fifty to sixty a minute were not uncommon, the extent of the pulsations becoming progressively less and less until it was almost impossible to tell whether the cells were pulsating at all, the erythrophores apparently remaining contracted. Usually after the erythrophores had ceased pulsating a period of quiescence set in lasting from three to five minutes, followed by a second and on occasion even a third or fourth period of activity. Thus the time in which the pulsatory activity could be seen usually lasted forty-five minutes to an hour and a half, although now and then individual cells were seen to pulsate for three hours or more, long after all the rest of the erythrophores in the scale had become quiet. During the first few minutes of the pulsations the movements of all the erythrophores as well as the xanthophores were synchronous throughout the scale. As time went on, however, the chromatophores comprising the pigmented area broke up into smaller groups, each group proceeding to pulsate at a rhythm independent of the others. This synchronism might indicate some sort of coördinating mechanism within the scale. The rate and extent of the erythrophore pulsations in one scale, however, compared with another were extremely variable, scarcely any two scales being even approximately alike.

The failure of the melanophores to pulsate under conditions which excite the erythrophores and xanthophores to activity does not indicate that these cells are incapable of such activity. Pulsations may be induced in the melanophores by use of a method originally introduced by Spaeth (1916). This requires a five-minute exposure of the cells to N/10 $BaCl_2$ followed by a transfer of the scale to N/10 NaCl. In *Holocentrus* scales so treated the melanophores show good pulsations in about five minutes after the immersion in NaCl following treatment with $BaCl_2$. These pulsations differ from those seen in the erythrophores and the xanthophores in that they are much slower, occurring only once or twice a minute. Slow pulsations of this sort are also characteristic of the melanophores of *Fundulus heteroclitus* in which such activity has been extensively studied (Spaeth, 1916; Smith, 1930).

Erythrophore pulsations have been reported before. In fact this

type of behavior on the part of chromatophores was first observed in connection with the erythrophores of *Mullus*. Ballowitz (1913) described rhythmical movements of the pigment granules in the erythrophores of excised bits of the skin of this form when such pieces of the skin are immersed in 0.75 per cent NaCl. Judging from his descriptions, erythrophore pulsations in this form were of the slow type seen in the melanophores of *Fundulus*. The isolated scale erythrophores of *Scorpæna ustulata* can also be made to pulsate (Smith and Smith, 1934) but only after they have been previously treated with $BaCl_2$. In this respect the *Scorpæna* erythrophores resemble more the melanophores of *Fundulus* and *Holocentrus* than the erythrophores of *Holocentrus*. In *Scorpæna* the erythrophores pulsate steadily for an hour or so at a rate of about once every two minutes. Melanophores in the same scale pulsate somewhat more rapidly, about once a minute on the average.

FIG. 3. Showing the progressive contraction of the chromatophores in N/10 KCl after sea water. Cells in the lower right contracted, cells in the upper left still expanded. The small bodies are erythrophores and the larger ones melanophores.

When *Holocentrus* scales are placed in N/10 KCl all of the chromatophores show a marked contraction (Fig. 3). This is quite in keeping with the results of other investigators upon the effect of KCl upon isolated scale chromatophores. In $CaCl_2$ the erythrophores and xanthophores of *Holocentrus* show a brief period of pulsatory activity lasting about eight minutes, after which they assume the contracted condition. That the pulsations were occurring at a time when the cells were under the influence of the $CaCl_2$ is shown by the fact that the iridocytes throughout the entire scale were colorless. Attempts were also made to study the effects of various drugs upon erythrophore pulsations but the results were not worthy of serious consideration since no adequate means of controlling the experiments could be found. Comparison between two scales, even adjoining scales from the same fish,

were quite useless as there was no constant uniformity in respect to the erythrophore behavior of the two. Scales were even cut into two halves, one half being immersed in solutions of the drug to be tested and the other half in NaCl as a control, but again no reliance could be placed on the data from a quantitative standpoint as control experiments showed that the erythrophores of the two halves of the same scale when placed in NaCl differed markedly in their behavior.

From these results and the results of previous investigation (von Frisch, 1912; Smith and Smith, 1934) it is apparent that in certain fishes the erythrophores are clearly under nervous control. In forms such as *Phoxinus* (Giersberg, 1930) it is equally clear that the erythrophores are independent of nervous control and are governed in their reactions by humoral factors. It still remains to be seen whether this bears any relation to the fact previously noted by us that in those forms where the erythrophores are under partial or complete humoral control their principal function seems to be their contribution to the assumption of the nuptial coloration shown during the spawning season. At other times they are relatively inactive. In forms such as *Holocentrus* and *Scorpæna* where a nervous control has been demonstrated the erythrophores are continually active at all seasons of the year and are in fact the dominating pigment cell among the chromatophores these animals possess.

In *Holocentrus* the erythrophores and xanthophores are alike in their behavior, both types of cells reacting about the same throughout all of the experiments attempted although possibly the xanthophores were somewhat more sluggish than the erythrophores in their responses to temperature changes. In *Scorpæna,* however, the xanthophores differ markedly from the erythrophores in that they show no pulsations in NaCl after treatment with $BaCl_2$ as do the erythrophores and are also much more sluggish in regard to their responses to other types of stimuli. Schnakenbeck (1925), after an examination of eighty different species of fish, came to the conclusion that there was no fundamental difference between xanthophores and erythrophores, in many cases both types of pigment being present in the same cell. On physiological grounds, however, such a generalization does not appear to be justified, although it may well be true of some fishes (i.e., *Holocentrus*). Any comparison between erythrophores and xanthophores other than in the same species seems useless until it is definitely established whether or not there are two distinct types of erythrophores, first the sluggish humorally controlled kind found in *Phoxinus* and second the highly sensitive nervously controlled type found in *Holocentrus.* Whether the difference between the two is actually clean cut, or whether the apparent

distinction is only illusory as a result of the study of two extreme types in a graded series is a matter for further investigation.

Further complexity is again suggested when the melanophores and the erythrophores are compared. In *Scorpæna* the two types of pigment cells seem to behave essentially the same, differing only on morphological grounds. In *Holocentrus,* on the other hand, the melanophores vary pronouncedly from the erythrophores not only in appearance, for this they do also in *Scorpæna,* but also in responsiveness to certain types of stimuli. In *Holocentrus* the melanophores do not pulsate spontaneously in NaCl when transferred directly from sea water. In this species the erythrophores were by far the most active type of pigment cell present in the scale. The melanophores appeared to be sluggish in comparison, although the melanophores of *Holocentrus* were no more sluggish in their behavior than the melanophores of other forms. But the extreme sensitivity of the erythrophores of *Holocentrus* might well be anticipated after witnessing the rapidity with which this animal can adapt itself to a new shade of background. Apparently this form possesses a pigment cell which is fully as responsive as the chromatophores of lizards and in fact approaches the sensitivity of the entirely distinct pigmentary effector organ of the cephalopod.

Summary

1. In the common Bermudian squirrel fish, *Holocentrus ascensionis* (Osbeck), sympathetic pigment-motor fibers can be demonstrated which are capable of altering the state of pigment distribution within the erythrophores.

2. In the denervated trunk erythrophores of *Holocentrus* an increase in temperature will cause a withdrawal of the pigment into the central body of the cell. Lowering the temperature has the opposite effect. In innervated trunk erythrophores the effect of temperature changes upon the state of pigment distribution of the erythrophore is the reverse of what is seen in denervated erythrophores.

3. Isolated scale erythrophores and xanthophores of *Holocentrus* show spontaneous pulsations when transferred from sea water to N/10 NaCl. Isolated scale melanophores will not pulsate in NaCl unless previously treated with N/10 $BaCl_2$.

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STUDIES OF LIVING NERVES[1]

IV. Growth, Regeneration, and Myelination of Peripheral Nerves in Salamanders

CARL CASKEY SPEIDEL

(From the Department of Anatomy, University of Virginia Medical School, University, Virginia)

In the earlier papers of this series of studies, the writer has presented observations of nerve activities as seen in living frog tadpoles (Speidel, 1932, 1933, and 1935). Case histories of individual nerve fibers over long periods of time have been obtained. These have revealed fundamental movements and changes that occur during the growth of nerves, during the formation of the myelin sheath, and during nerve irritation, degeneration, and repair.

Up to the present time frog tadpoles are the only forms in which nerve changes have been seen by this method of direct observation *in vivo*. It seemed desirable that a similar study be made on some representative of another group of vertebrate animals. Preliminary observations on a number of species of urodele amphibians and fishes indicated that it was possible to study living nerves in some of these. The present investigation deals with the behavior of the peripheral nerves in the tail of young salamanders during growth, regeneration, and myelination.

Material and Method

The best observations were obtained on young salamanders (*Triturus viridescens*) during the fall of the year. These were approximately seven or eight months old. An average specimen was 30 mm. in length. Animals of other ages, however, were also watched. These included young larvae recently hatched from the eggs, and a few animals more than a year old.

The animals were examined microscopically under light chloretone anesthesia in an upright paraffin chamber. Between observations they were kept isolated in jars containing pond water and vegetation conducive to growth. The finer details of structure were observed with the aid of a 3-mm. oil-immersion objective lens.

[1] This work has been aided by grants from the National Research Council, from the Committee on Scientific Research of the American Medical Association, and from the Research Committee of the Virginia Academy of Science.

Distribution of Nerves in the Tail of the Salamander

The central portion of the tail is occupied by the spinal cord, axial skeleton (notochord), and the muscle masses. This region is not suitable for observation in the living animal. The spinal cord extends almost to the tip of the tail. Emerging from between the muscle masses may be seen the cutaneous nerves that supply the tail fin expansion. Many of these are of the mixed type consisting of both myelinated and unmyelinated fibers. Others are entirely of the unmyelinated type. Occasionally, a single myelinated fiber may be seen which is isolated from other fibers for a part of its extent. Myelin-emergent sprouts are often intimately intermingled with non-myelin-emergent fibers. Sometimes, however, they are separate and can be traced to their terminations just beneath the basement membrane of the epithelium.

The lateral line nerve and its branches constitute a conspicuous part of the peripheral nervous system in the tail. Rami of this nerve, which is itself a division of the vagus nerve, are particularly prominent in the dorsal fin region. These fibers innervate the many lateral line organs of special sense which are located in the tail.

Essentially, the peripheral nervous system of the tail of the young salamander is like that of the frog tadpole. A few minor differences, however, may be noted. In the salamander the sheath cells of Schwann are larger, the myelin segments longer, and the myelinated fiber is of smaller caliber; all of the regenerative activities proceed at a slower rate; the sheath cells migrate and proliferate less rapidly; the ameboid growth cones travel at a slower pace; and the process of myelin ensheathment requires more time.

Growth of Individual Nerve Sprouts and the Formation of the Early Unmyelinated Nerves

Pioneer Growth Cones

Experimentally many actively growing nerve sprouts may be induced by subjecting the animal to partial tail amputation followed by several days of regeneration. Careful examination of the newly regenerating zone reveals the expanded growing tips of single nerve fibers. These terminal enlargements are called growth cones. Each pioneer growth cone probes its way slowly through the mesenchymal tissues spinning a nerve fiber behind it. The growth cones migrate by ameboid movement. Delicate pseudopods are frequently extended and retracted. At times tiny refractive granules are visible.

Progress, though rapid at times, is somewhat sporadic. Slight barriers, such as connective tissue cell processes, may cause temporary halt-

ing and varicosity formation. A change in the direction of growth of the nerve fiber may follow. Growth cones at rest usually draw in their pseudopods and become smoothly rounded. Retracting nerve tips are characterized by the appearance of knob-like excrescences or vesicles.

Typical behavior of a pioneer growth cone over a period of one hour is shown in the illustration (Fig. 1). Extension, temporary varicosity formation, constriction ring formation, deflection by a connective tissue cell process, and variations in pseudopods and in granular content are all visible in this example. A distance of about 20 micra was traversed during the hour.

The group of pioneer growth cones shown in the illustration (Fig. 2) exemplifies another principle which is of some theoretical interest. In spite of the close proximity of the growth cones to one another with presumably very similar local environment, their behavior varies. While some exhibit definite retraction, others actively advance or show no marked change in position. This suggests that there must be a distant central influence over nerve sprout advance; that, though local conditions may be important, they are not the sole determiners of the behavior of the sprouts.

In one of the other case histories (Fig. 4) the behavior of several growth cones over a period of three days is shown. This case suggests

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PLATE I

The following figures are based upon free-hand sketches, and they are, therefore, not entirely accurate. In all figures *proximal* is toward the left (or below), and *distal* toward the right (or above).

FIG. 1. The progress of a growth cone of a nerve fiber over a period of one hour. Salamander, No. 882, regenerating zone eight days after partial tail amputation.

11:30 A.M., the growth cone, *S*, is slightly obstructed by the fibroblast process, *P*. 11:37 to 11:40, the growth cone passes the obstruction, a constriction ring being present at the point. 11:51 to 12:08, a second connective tissue cell process is passed which is located somewhat obliquely to the course of growth of the nerve fiber. 12:25 to 12:30, a third process of a connective tissue cell is approached.

FIG. 2. Movements of extension and retraction shown by several free growth cones within a small area. Salamander, No. 881, eight days after partial tail amputation.

Five growth cones are visible at *P*, *Q*, *R*, *S*, and *T*. During thirty-five minutes of observation growth cones *P*, *R*, and *T* advanced somewhat, while *Q* and *S* both retracted.

FIG. 3. The extension of the first three growth cones of a young nerve over a period of one hour. Salamander, No. 882, regenerating zone, eight days after partial tail amputation.

At 11:35 two growing tips were visible at *P* and *Q* advancing slowly through the tissues toward the edge of the tail fin. A third growth cone at *R* was diverging slightly from the line of the nerve.

12:35, one hour later *P*, *Q*, and *R* all showed some progress and at *S* a new growth cone appeared.

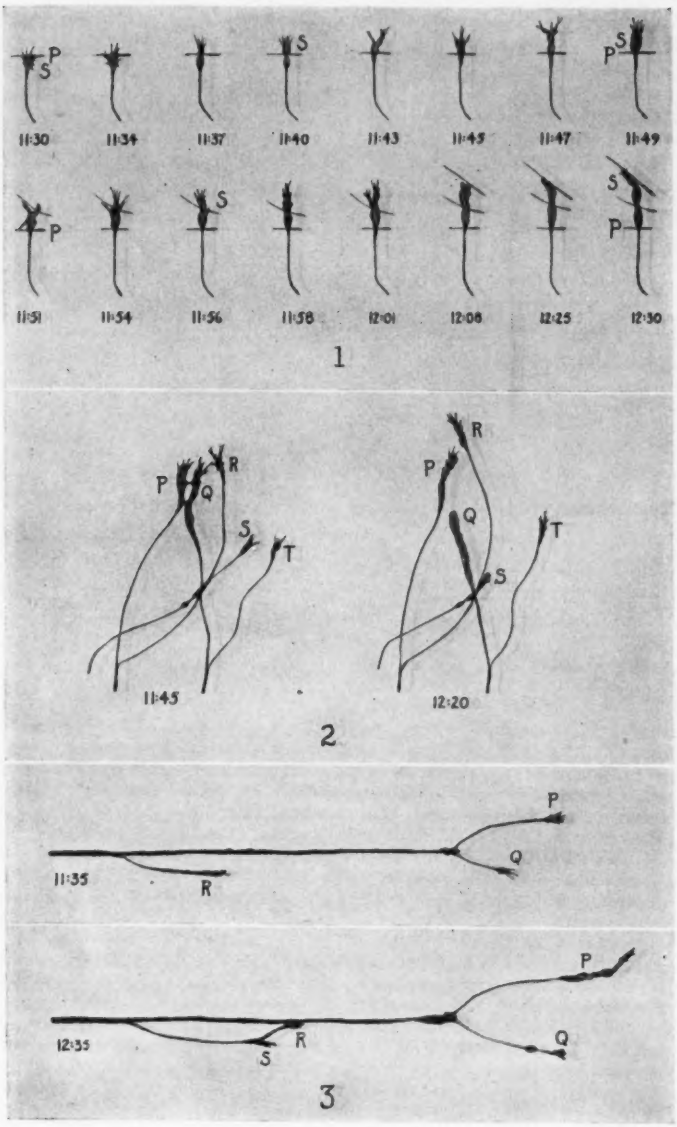
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PLATE I

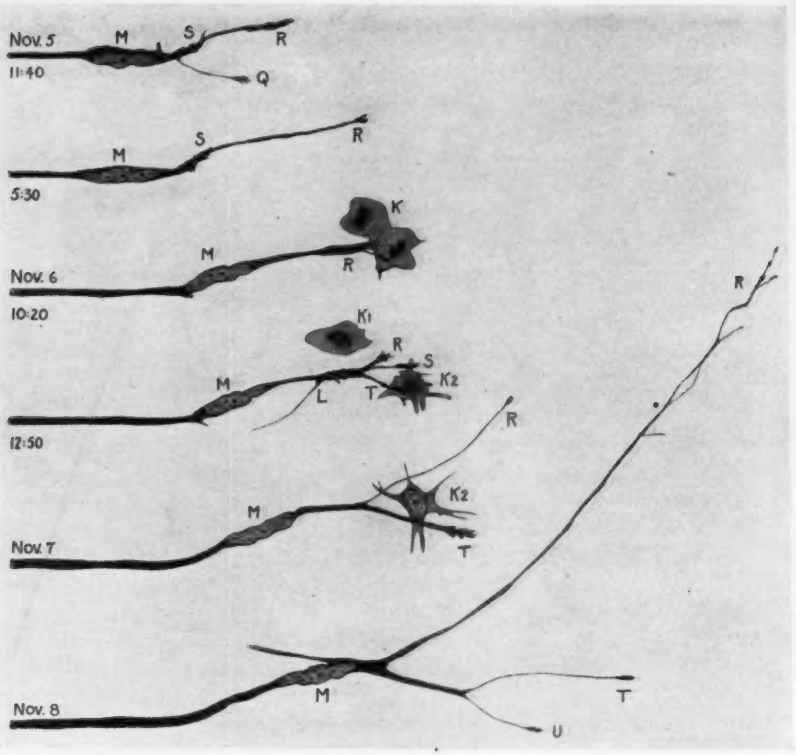

PLATE II

FIG. 4. Movements of growth cones and a sheath cell of Schwann over a period of three days; the formation of branches correlated with a nearby fibroblast mitosis. Salamander, No. 881, regenerating tail fin observed from eight to eleven days after partial tail amputation (cut October 28).

November 5, 11:40 A.M., three growth cones, *Q*, *R*, and *S* are visible. The primitive sheath cell, *M*, has reached the position shown and exhibits slow movements with little change in position.

November 5, 5:30 P.M., the small branch which ended in *Q* has disappeared. *R* has advanced.

November 6, 10:20 A.M., sheath cell *M* has advanced. *R* is swollen and in close relation to the dividing fibroblast, *K*. Growth cone, *S*, is indistinguishable but is probably present in the swollen tip at *R*.

November 6, 12:50 P.M., three separate growth cones, *R*, *S*, and *T* advance as the cell division becomes completed with the formation of the daughter cells, *K*1 and *K*2. Another growth cone touches the nerve at *L*, causing some agitation of the neuroplasm at this point.

November 7, *R* and *T* have advanced, but *S* has disappeared. The connection at *L* has also been lost. The position of *K*2, one of the daughter cells of the fibroblast division of the preceding day, is shown.

November 8, *M* advances somewhat. And at its distal end a new connection with another nerve is present. Growth cones, *R* and *T*, show further extension and a new branch develops which ends in the growth cone at *U*. The nerve increases in diameter.

that mitosis of an adjacent fibroblast has a stimulating effect on nerve sprouts. It also indicates that the neuroplasm of a young nerve is responsive to the touch of an active growth cone. Similar observations have been recorded in the case of frog tadpoles (Speidel, 1933 and 1935).

Later Growth Cones

Following the first nerve fibers come the second, third, and later growth cones, each spinning a fiber of its own. These usually follow more or less closely the pathway laid down by the pioneer growth cones. In this manner the first small unmyelinated nerves are formed.

In the example given (Fig. 3) a small regenerating nerve is shown which toward the left consists of three fibers. The terminal growth cones of these fibers are visible at *P*, *Q*, and *R*. Over a period of an hour each of these advances somewhat. The origin of a new sprout from one is visible as a new growth cone forms at *S*.

The Primitive Sheath Cells of Schwann

The early small nerves which at first consist of only a few naked fibers become quickly provided with satellite cells, the primitive sheath cells of Schwann (neurilemma cells).[2] These cells migrate distally along the nerves by ameboid movement. The example given (Fig. 4) shows the rather slow progress of the most peripheral sheath cell on a small nerve over a period of three days.

In regenerating zones the Schwann cells often divide by mitosis (Fig. 5). While at rest these cells are much elongated and flattened along the nerve. In preparation for division, however, they slowly swell and round up. The fast motion ciné-photomicrographs vividly reveal the violent agitation that takes place during the actual division into two cells. Distinct local swelling of the nerve usually accompanies the process. The daughter cells then move apart and slowly flatten out to assume their typical elongated resting position along the nerve.

A sheath of Schwann (neurilemma), syncytial in nature, is elaborated. This sheath is tubular and at first usually encloses *several* nerve fibers. Septa may develop later to effect separate ensheathment for single fibers.

The Process of Myelin Sheath Formation

As nerve fibers become more mature many acquire a myelin sheath. This sheath is laid down in segments. The first segments appear near

[2] Embryologically, the sheath cells of Schwann are derived from ectoderm. They originate from the neural crest of the developing spinal cord, migrate outward along the dorsal roots, and then follow the spinal nerves.

the nerve roots. The progress of myelination is from proximal to distal. Each new segment is added to the end of the myelin line. As in frog tadpoles, a nerve fiber emerging from a myelin segment (myelin-emergent fiber) exhibits a greater bias toward myelin formation than one which does not emerge from a myelin sheath (non-myelin-emergent).

Myelin-emergent and Non-myelin-emergent Fibers

Careful examination of nerves near the transition between a proximal myelination zone and a distal non-myelination zone reveals that myelin-emergent sprouts may sometimes be distinguished from the non-myelin-emergent fibers which make up the bulk of an ordinary unmyelinated nerve. In the example given (Fig. 6) extremely delicate myelin-emergent sprouts emerging from the last myelin segment are visible. They are quite distinct from the fibers of the accompanying unmyelinated nerve which is provided with a sheath cell of Schwann and a neurilemma. The myelin-emergent sprouts are without a sheath of any sort and are, therefore, quite naked. In this particular instance no further myelination took place although the region was observed for 60 days.

As a rule, however, a myelin-emergent fiber which is ripe for ensheathment is intimately associated with accompanying non-myelin-emergent fibers, often being within the same neurilemma at first. For this reason, it is ordinarily not distinguishable. In all of the following examples it is noteworthy that each new myelin unit is formed as a result of the coöperative activity of a sheath cell and a nerve fiber, particularly a myelin-emergent fiber.

Addition of Two Terminal Myelin Segments

In the example (Fig. 9) a small mixed nerve consists of a single fiber in process of myelination and an unmyelinated nerve which is made up of several nerve fibers. To the right of the arrow in the direction of the young sheath cell *S*, the unmyelinated nerve includes both a myelin-emergent fiber and several non-myelin-emergent fibers. Proliferation of the primitive sheath cells is followed closely by the formation of two new myelin sheath segments. Marked swelling of the myelin-emergent fiber accompanies the process.

Comparison of the young myelin segments in the last two sketches brings out the slow but steady growth in both diameter and length of the myelin units.

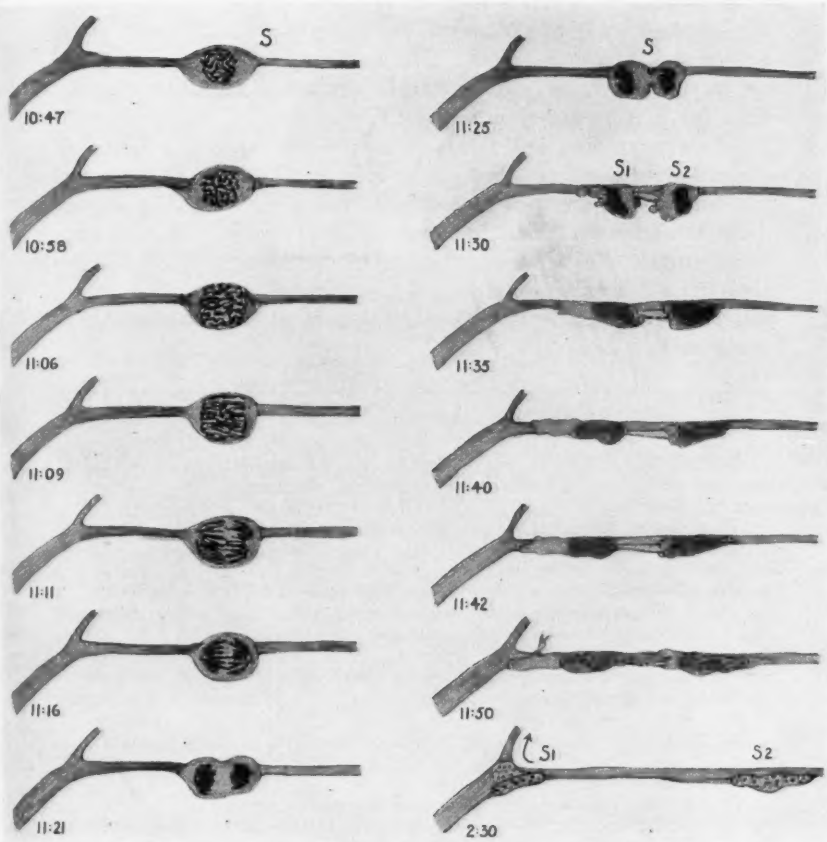

PLATE III

FIG. 5. Sheath cell division on a small peripheral unmyelinated nerve. Salamander, No. 880, regenerating tail fin ten days after partial tail amputation.

During the hour from 10:47 A.M. to 11:50 A.M. the changes in a dividing sheath cell are shown. At 11:51 the animal was replaced in pond water. At 2:30 P.M. the two daughter cells were separated as shown. During the next day the cell, *S*1, migrated out of the upper fork of the nerve in the direction indicated by the arrow.

Intercalation of a Myelin Segment Between Two Others

Sometimes myelination along a fiber takes place somewhat irregularly, in such a way that temporary bare lengths are left in some places between two myelin segments. Such bare lengths of fiber, if they are not very extensive, are usually covered by growth of the adjacent myelin units (cf. Fig. 10, growth of segment *T* toward *U*).

If the unmyelinated portion is too extensive, however, the process of ensheathment is accomplished by the intercalation of an entire myelin unit with its own sheath cell. In the example given (Fig. 10) between the myelin units at *R* and *T* is a region without myelin. Since a primitive sheath cell *S* is located in this region, conditions are favorable for myelination. Within two days a young segment is intercalated which then extends during the next two days to reduce the unmyelinated gaps at each end.

PLATE IV

FIG. 6. A small mixed nerve showing the intimate relation of a myelin-emergent fiber with the accompanying non-myelin-emergent fibers. Normal salamander, No. 878.

From the terminal myelin segment, *T*, emerges a fiber which divides into naked sprouts at *S*. One of these is visible as it extends in close association with the accompanying unmyelinated nerve toward the sheath cell, *R*. Several of the sprouts terminate immediately beneath the basement membrane of the skin epithelium.

FIG. 7. Rapid swelling and ovoid segmentation of a segment of the myelin sheath following a hot water burn. Salamander, No. 997.

12:15, a normal myelin sheath segment. At 12:18 the tip of the tail, close to the segment illustrated, was immersed in hot water for a few seconds.

12:20, both the myelin segment and the sheath cell nucleus have become greatly swollen.

12:22, the sheath breaks up into myelin ovoids which remain connected for a time.

12:30, a later stage.

FIG. 8. Appropriation of a portion of one myelin segment by the next one following end-to-end anastomosis and establishment of a new node of Ranvier. Regenerating zone of salamander, No. 867, following tail section on October 12. The segments of this figure are the same ones illustrated in Fig. 12, segments *N*, *O*, and *P*.

November 24, a bare length of fiber without a myelin sheath lies between the myelin segments associated with *O* and *P*.

December 6, sheath cell, *O*, moves toward the bare length. Both segments grow in length.

December 19, the myelin segments become joined. Thus, two sheath cells are located for a number of days on a single fused segment.

January 6, sheath cell, *P*, migrates toward the right beyond the field illustrated and a new node of Ranvier develops between *O* and *P*. The myelin sheath between the two arrows *originated* under the influence, not of sheath cell *O*, but of sheath cell, *P*.

During the same period a new terminal segment forms under the influence of $Q1$ along the upper nerve fork, following a division of the young sheath cell Q. Movements of sheath cells, N, P, and $Q2$ are also evident over the 4-day period illustrated.

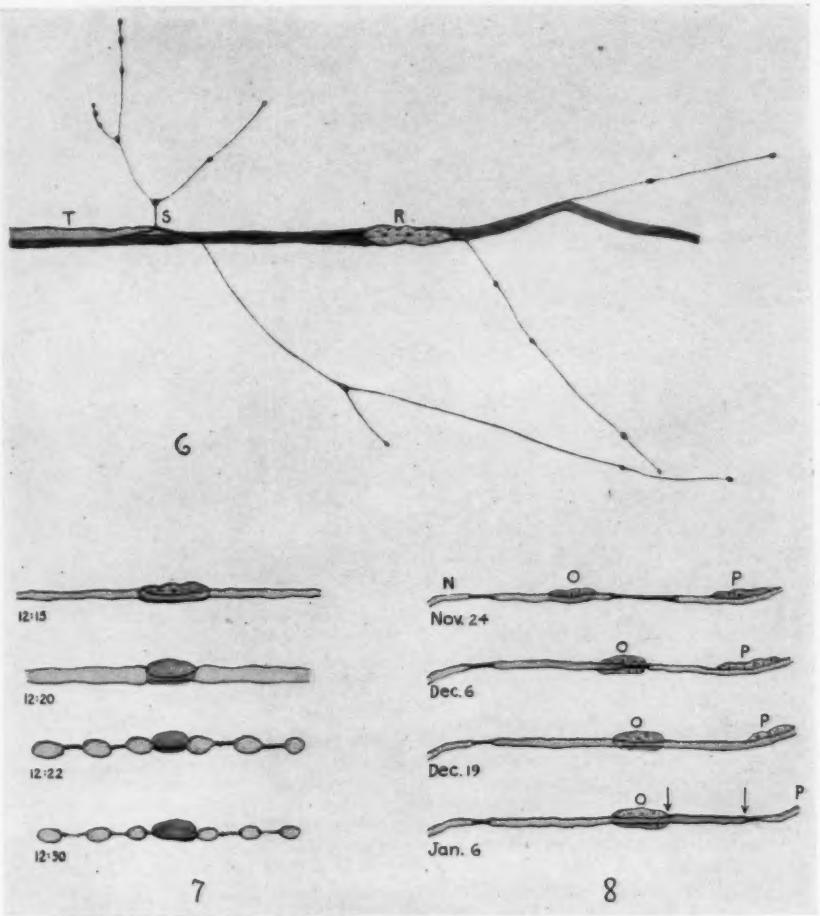

PLATE IV

Appropriation of a Part of a Myelin Segment by the One Next to It, Following an End-to-end Anastomosis of the Two Segments

Occasionally, myelin is elaborated at the primitive node of Ranvier between two young segments. This results in fusion of the two seg-

ments with complete obliteration of the node (Fig. 8). The elongated segment is left for a time with two sheath cells. In the example cited, one of the sheath cells moved distally for a short distance and a new

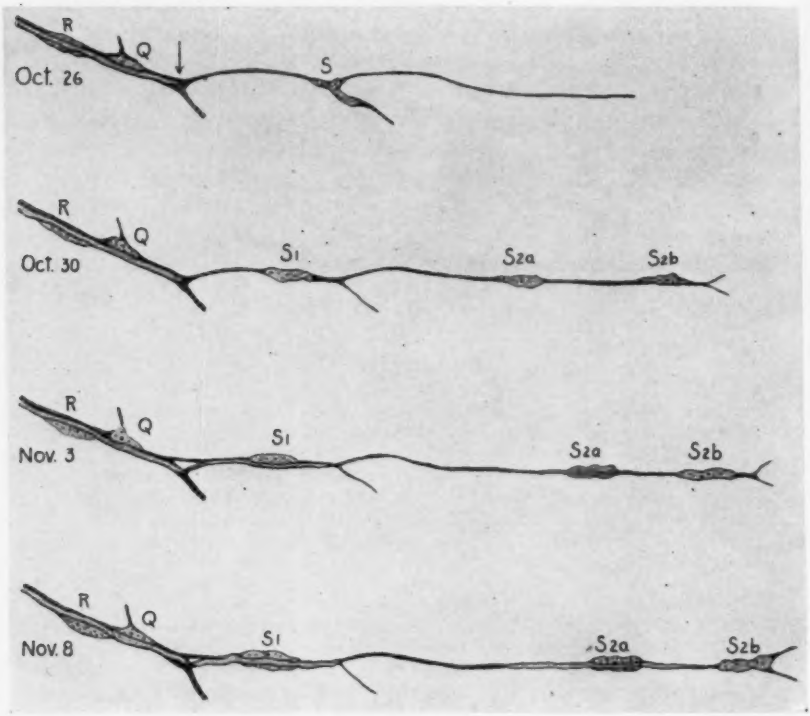

PLATE V

FIG. 9. Addition of two new myelin sheath segments at the end of a myelinating fiber in a normal young salamander, No. 869.

October 26, the myelin sheath of a single myelinated fiber ends at the point indicated by the arrow. The sheath cell, *R*, is associated with the final myelin segment. The sheath cell, *Q*, is on the accompanying unmyelinated nerve. The most peripheral sheath cell on the nerve is shown at *S*.

October 30, *S* divides once, giving rise to *S*1 and *S*2 and the latter daughter cell divides giving rise to *S2a* and *S2b*.

November 3, two new myelin segments are formed, one associated with *S*1 and the other with *S2a*.

November 8, the new segments grow somewhat both in length and in diameter and in thickness of the myelin sheath.

This region was kept under observation for more than two months longer. During this period the animal was subjected to partial starvation and no further essential growth changes took place. Finally, on January 18, the myelin segment associated with *S2a* suffered degeneration, possibly as an indirect result of malnutrition.

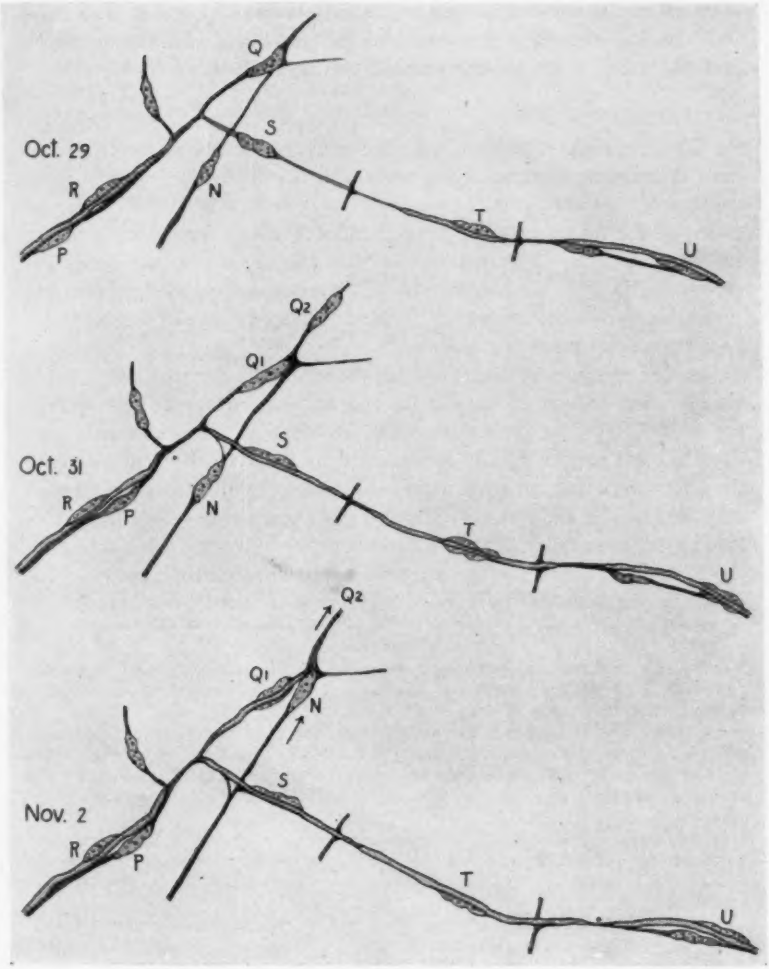

PLATE VI

FIG. 10. Intercalation and addition of new myelin sheath segments in the vicinity of a nerve fork. Normal salamander, No. 876.

October 29, three myelin segments are visible associated with the sheath cells, *R*, *T*, and *U*, respectively. Sheath cells, *P*, *Q*, *S*, and *N* are associated with unmyelinated nerve fibers.

October 31, a new myelin segment appears in relation with *S*. Longitudinal extension of the myelin sheath segments associated with *R* and *T* is also noticeable. *Q* divides, giving rise to *Q*1 and *Q*2.

November 2, a new myelin segment is formed in relation with *Q*1. The segment associated with *S* grows both in length and in diameter.

node of Ranvier then appeared at a locus previously covered with myelin. In this manner a segment was left provided with myelin which had originated under the influence of two sheath cells.

Rapid Myelinogenesis During Regeneration

The preceding examples of myelin segment formation are from animals undergoing slow normal growth and development. A very rapid process of myelination may be induced experimentally by partial amputation of the tail, followed by regeneration. From the cut ends of the nerves at the site of amputation sprouts grow out into the newly regenerating tail tip, the sheath cells of Schwann follow along them, and typical unmyelinated nerves are formed. Lines of myelin segments appear later along some of the fibers.

In the example given (Fig. 12) a region is shown in the newly regenerating tail fin 11 days after the amputation operation. During the next 7 days the nerves increased in size and the sheath cells proliferated and moved distally as indicated by the arrows. That some of the fibers were ripe for myelination is indicated by their swollen appearance and by the alignment of sheath cells along the edge. On the following day many new definitive myelin segments were visible. During the next three weeks further progress of myelination took place. This consisted not only in the formation of new segments, but also in the

PLATE VII

FIG. 11. Pressure irritation and degeneration of terminal myelin segments, followed by temporary restoration of one and loss or transfer of sheath cell. Normal salamander, No. 866.

October 23, at the end of a single nerve fiber four normal myelin segments are visible associated with their sheath cells, *Q*, *R*, *S*, and *T*, respectively. On October 26, as a result of slightly too much pressure exerted by the cover slip during observation, these segments became greatly irritated and degeneration ensued.

October 30, sheath cell, *T*, disappears entirely. Sheath cell, *S*, has transferred to a near-by nerve. Sheath cells, *Q* and *R*, remain in position. Small myelin ovoids and granules are in evidence along the degenerating fiber. New sprouts appear at *N* and at *J*.

November 3, in association with sheath cell, *Q*, a new myelin segment forms. It grows in length during the next few days. Sheath cell, *S*, migrates farther distally along the nerve, as indicated by the arrow. On November 6 the sheath cell, *R*, becomes somewhat vacuolated and then undergoes degeneration. On November 12 the newly regenerated myelin segment associated with *Q* degenerated.

November 16, sheath cell, *Q*, transfers to a near-by nerve and slowly migrates in the direction of the arrow. A new myelin segment appears in association with *L*.

December 12, a sheath cell, *M*, transfers from the nerve at the right and moves as indicated by the arrows, finally arriving at the node of Ranvier between segments, *K* and *L*. A second line of myelin segments appears on one of the fibers of the mixed nerve at the right of the figure. The wrinkling of the myelin sheath of the segment associated with *K* is indicative of an irritated state. On January 3 this segment degenerated.

growth in length and diameter of each unit, and increase in the thickness of the myelin.

In the large nerves the intimate intermingling of the fibers in

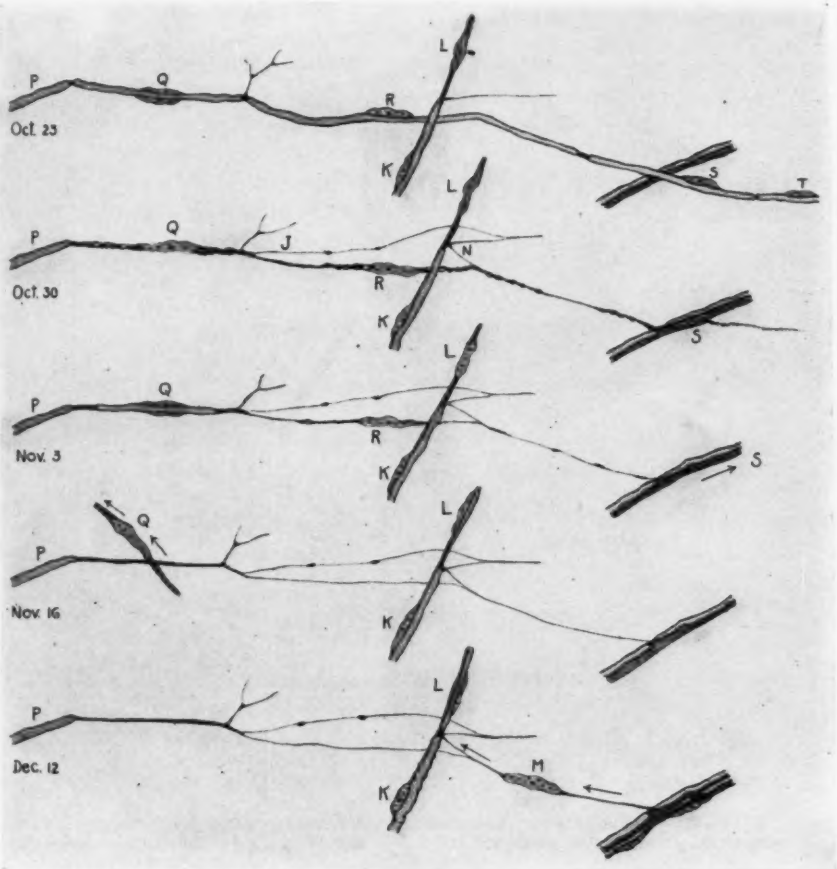

PLATE VII

process of myelin ensheathment with those that remained unmyelinated prevented clear-cut histories for all segments. In some cases, however, the complete history of formation and growth of single segments is obvious (as with *M*, *R*1, *T*, and *U*).

This example of regeneration, together with others of similar nature,

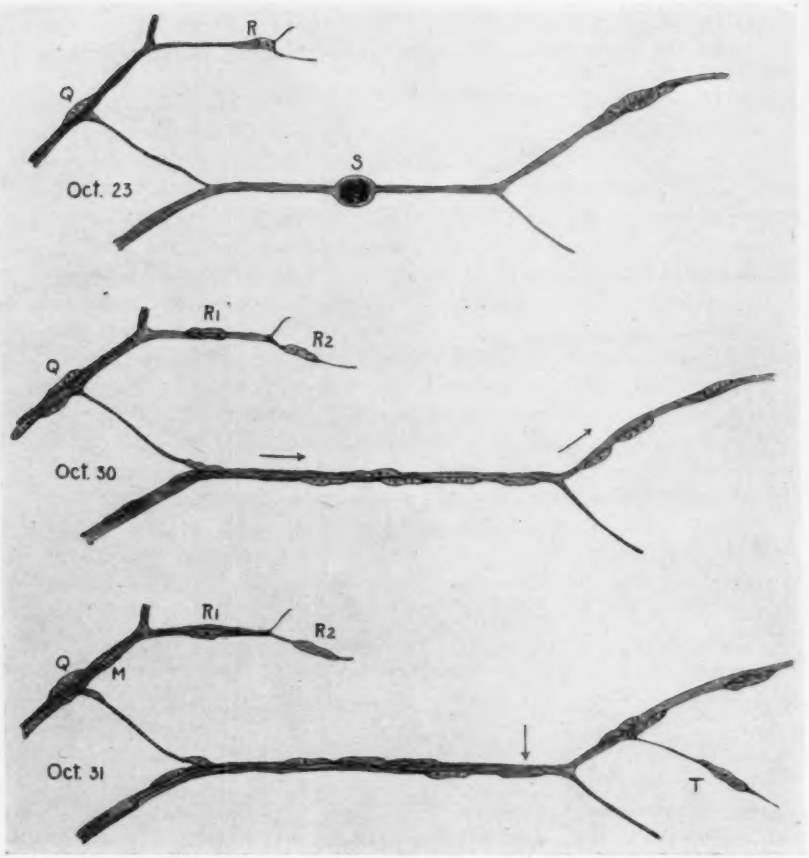

PLATE VIII (A)

FIG. 12. Myelinogenesis during rapid regeneration. Salamander, No. 867, regenerating zone, 11 to 40 days after partial tail amputation. The tail was sectioned on October 12.

October 23, a sheath cell, *S*, on a newly regenerating unmyelinated nerve is in mitosis. Two other sheath cells are visible on this nerve distally. The mixed nerve at the upper left of the figure includes an unmyelinated portion and a single myelin segment associated with the sheath cell at *Q*.

October 30, the sheath cells multiply and exhibit migration distally, as indicated by the arrows. Sheath cell, *R*, divides into *R*1 and *R*2. Both nerves have a somewhat swollen appearance and there is a distinct tendency for the sheath cells to align themselves along the nerve edges.

October 31, two lines of thin myelin segments become differentiated in the lower nerve extending distally to the point indicated by the arrow. In the upper nerve a second fiber becomes provided with a segment appearing at *M* and another at *R*1. A new sprout and sheath cell appear at *T*.

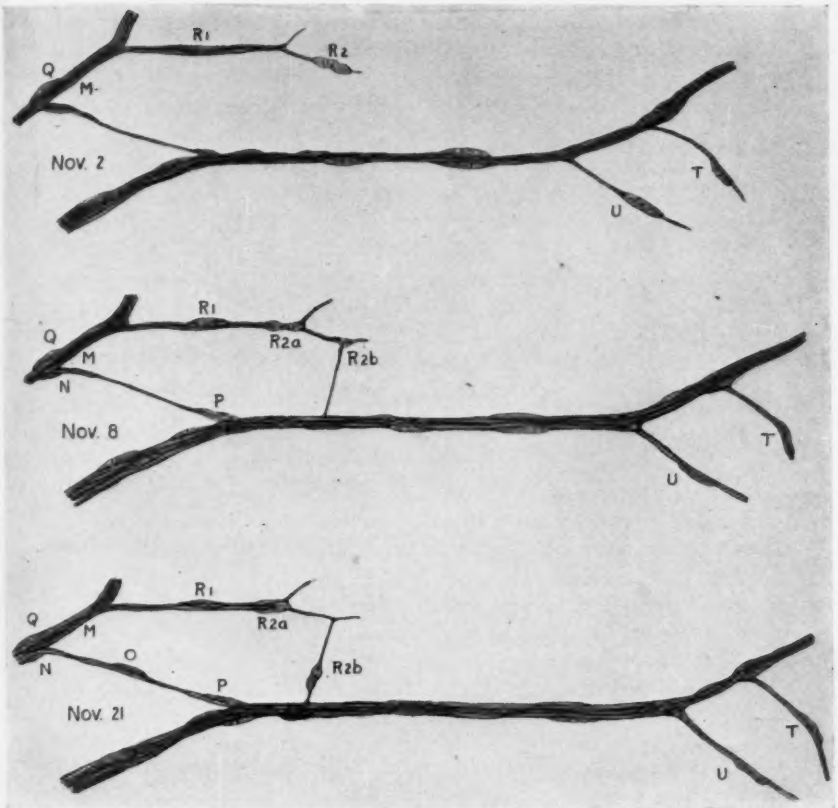

PLATE VIII (B)

November 2, myelin segment formation proceeds distally. A new segment is formed at *T*.

November 8, the myelinated fibers become more conspicuous and many of the separate segments exhibit growth in length and thickness. New myelin segments are visible at *U* and at *N*.

November 21, the lower nerve now includes three myelinated fibers as well as an unmyelinated portion. Elongation of segments takes place which is correlated with the growth of surrounding tissues as regeneration of the tail proceeds. New myelin segments are visible at *O* and *P*.

This region was kept under observation until January 18, during which time some further minor changes took place. Segments, *O* and *P*, presented a history of special interest, which is shown in Plate IV, Fig. 8.

makes it clear that the great time for new myelin segment formation in the proximal regenerating zone is from about 18 to 25 days after the injury. In more distal zones of regeneration this may be somewhat later.

This case also brings out how whole nerves lengthen or stretch to keep pace with the growth of the terrain. Thus, the distance between the two forks of the lower nerve becomes distinctly greater during the 29 days represented by the sketches (Fig. 12).

Myelination of a Cranial Nerve

Branches of the lateral line nerve (a vagus nerve division) were watched to see whether there might be any difference in the mechanism of myelin-sheath formation along a cranial nerve fiber. Case histories of a few myelin segments were obtained which indicated that the process of myelination on this cranial nerve is entirely like that observed on the spinal nerves.

Nerve Irritation and Adjustment

During growth and regeneration the nerves are subjected to various stresses and strains. Nerve irritation is frequently the result. A condition of this sort is easily recognizable because of the reaction exhibited by the myelinated fibers.

An irritated internodal segment may exhibit swelling, vacuole formation between the axis cylinder and the myelin sheath, widening of the node of Ranvier as the myelin sheath slips or disappears in this region, and wrinkling of the myelin sheath. Myelin spherules may become detached from the sheath. If the irritation is strong the segment will degenerate, the sheath segmenting into myelin ovoids and granules. If the irritation is not too strong the segment may readily recover within a day or two, suffering little or no loss of its myelin sheath.

A beautifully graded series of irritated segments may be observed immediately after the tip of the tail is momentarily immersed in hot water. A nerve fiber which innervates the scalded zone shows acute irritation. On such a fiber the myelin segments near the edge of the burned region quickly swell and undergo rapid metamorphosis leading to the formation of myelin ovoids (Fig. 7).

Farther proximally the myelin segments are less strongly irritated. They exhibit a distinct swelling like that shown by the segment in the illustration (Fig. 7, at 12:20). They do not break up, however, and during the next few days, as recovery takes place, the swelling subsides.

Similarly, following partial tail amputation the myelin segments bordering the cut edge undergo traumatic irritation and degeneration.

Those farther proximally may remain quite intact and merely exhibit swelling. A case of traumatic degeneration following an injury to the overlying skin is given (Fig. 11). This case also illustrates replacement of one of the degenerated myelin segments, followed later by a second degeneration.

Whenever tissue shrinkage occurs, the nerve fibers in the zone of shrinkage become somewhat wrinkled. Sometimes a slight degree of telescoping of one segment on the next is evident. Swelling, vacuole formation, and detachment of small myelin spherules may accompany this condition of irritation. Recovery may, or may not, take place without loss of the myelin sheath, depending upon the strength of the irritation. Older more massive segments of the myelin sheath offer stronger resistance to the degenerative process than do younger slighter segments.

Typical phenomena of nerve irritation may also be observed after subjection of the animal to alcohol intoxication, to a strong anesthetic (chloretone), to pressure, to partial desiccation, to starvation or malnutrition, and to near-freezing temperature. Since many varieties and many gradations of nerve irritation of both acute and chronic types have already been described in some detail in the case of frog tadpoles (Speidel, 1935), it is unnecessary that further consideration be given to this subject in the salamander. Fundamentally, the reactions in both species are quite similar.

Degeneration and Regeneration Along the Proximal and Distal Stumps of Sectioned Nerves

After nerve section the processes of degeneration and regeneration in the salamander proceed as in the frog tadpole. Typical trophic (Wallerian) degeneration takes place along the distal stump. In the case of sectioned myelinated fibers the peripheral portion always exhibits total degeneration. A certain amount of traumatic degeneration is visible on the proximal stump close to the site of the wound. Repair is effected by growth of sprouts from the cut nerve fibers, by migration and multiplication of the sheath cells of Schwann, and finally by formation of new segments of myelin sheath. Ordinarily, the regenerating fibers follow the distal stump more or less closely.

The regeneration of sectioned unmyelinated nerves is more difficult to follow exactly. These nerves form anastomoses freely with one another. The distal stump of a cut unmyelinated nerve usually exhibits partial but not total degeneration. The explanation of this result, together with illustrative case histories, has already been advanced in con-

nection with similar results in frog tadpoles. The distal stump of such a nerve includes at least one *reverse* fiber which has not been severed from its cell body by the cut, but is still connected with it by way of a peripheral anastomosis with a normal unsectioned nerve. As far as such a reverse fiber is concerned the distal stump is in reality a proximal stump. Furthermore, after the operation other fibers from adjacent nerves may join the degenerating distal stump and grow along it in either direction. Junction of distal and proximal stumps may take place with growth cones progressing in either direction across the wound zone. Collateral sprouts are usually stimulated to grow out from the near-by uninjured nerves.

Mixed nerves, likewise, since they include an unmyelinated portion, may exhibit incomplete degeneration as far as the unmyelinated fibers are concerned.

Polariscopic Observations

Under the micropolariscope with crossed Nicol prisms the myelin sheath is brilliantly illuminated. It is negatively anisotropic. The neurilemma, axis cylinder, the unmyelinated fibers, and the terminal growth cones are all isotropic. Occasionally, though not often, one or more anisotropic granules may be present within a sheath cell.

In the development of a myelin segment the pre-myelin substance is isotropic. Very young myelin segments exhibit thin lines of weak anisotropy. Older and thicker myelin segments exhibit strong anisotropy. Thus, the development of the anisotropic condition closely parallels the growth of the sheath.

During myelin sheath degeneration, the anisotropic condition is exhibited by the myelin ovoids and granules. Gradually the anisotropy becomes less pronounced as degeneration proceeds. In the case of individual globules or granules, however, it may persist for several days or more.

Ciné-Photomicrographs of Nerve Fibers in the Salamander

Successful motion-picture records have been obtained of salamander nerve fibers showing the essential changes during growth and myelination. The magnification is sufficient to reveal some of the finest histological details. These ciné-photomicrographs are mostly of the fast-motion (time-lapse) type.

Movements of the growing nerve tips are vividly revealed by pictures taken at the rate of one every two seconds. When these are projected on the screen at the rate of sixteen per second all movements

become magnified thirty-two times. Movements of the sheath cells are quite slow and are best brought out on the screen by photographs taken at a slow rate, e.g., one every eight seconds. In projection these movements become magnified one hundred and twenty-eight times. For records of the daily progress of myelin sheath formation on a fiber, the pictures were usually taken at the normal rate of sixteen per second.

Among the subjects photographed are the following:

1. Rapidly advancing pioneer growth cones in newly regenerating regions (several examples).
2. Varicosity formation following the blocking of an active growth cone by connective tissue cell processes.
3. Movements of sheath cells of Schwann along newly regenerating nerves.
4. Mitoses of sheath cells of Schwann.
5. Addition of new terminal segments of myelin sheath along a regenerating nerve fiber.
6. Formation of many new myelin segments and the process of transformation of a composite unmyelinated nerve into a mixed nerve including several myelinated fibers. (Photographed at intervals from 16 to 71 days after partial amputation of the tail.)
7. Examples of strongly irritated myelin segments.

Summary

In living salamanders (*Triturus viridescens*) individual nerve fibers have been directly observed for prolonged periods (several months) during the processes of growth and myelination, degeneration and regeneration, irritation and recovery. The behavior of nerves in the young salamander is fundamentally like that in the frog tadpole.

In newly regenerating zones a few days old the earliest nerve sprouts are visible slowly probing their way through the mesenchymal spaces toward the skin. At the tip of each fiber is an enlargement, or growth cone, which advances by ameboid movement. In action the growth cone is provided with delicate pointed pseudopods; while at rest it is smoothly rounded; during retraction it is characterized by knob-like excrescences.

Growth of the sprouts is not necessarily continuous, but is often sporadic in nature. That local conditions are not wholly responsible for growth cone progress is suggested by the differences in behavior exhibited by closely grouped growth cones subject to approximately the same local environmental influences.

Barriers to growth cone progress are afforded by the tissues of the tail, such as the processes of connective tissue cells. Such barriers often induce retraction followed by some change of direction of growth cone extension. A varicosity may be left at a site of temporary obstruction.

Cell mitosis, *per se*, either of sheath cells or of fibroblasts, probably has a stimulating effect on nerve sprouts. An active growth cone on touching a small nerve may also induce neuroplasmic movements at the point of contact.

The second, third, and later growth cones usually follow more or less closely the pathway laid down by the first growth cone. Thus, the early unmyelinated nerves are formed. These are soon provided with sheath cells of Schwann, which migrate outward from the central regions and multiply by mitosis. These cells are necessary for the formation of both neurilemma (sheath of Schwann) and the myelin sheath.

The myelin sheath is formed only through the coöperative activity of the nerve fiber and the sheath cell. Not all nerve fibers, however, are equally ripe for myelination. Those which emerge from a myelin sheath (myelin-emergent) are especially ripe for the production of myelin. The differential factor, therefore, responsible for myelin is inherent, not in the sheath cell, but in the nerve fiber.

The myelin is laid down in segments, one segment genetically corresponding to the zone of influence of one sheath cell. The earliest myelin of a segment usually appears near the sheath cell nucleus. It grows by continuous extension in both directions away from the nucleus.

The first segments appear proximally near the nerve roots. Progress of the sheath is in the distal direction, each new segment being added at the end of the myelin line.

Myelination in salamanders usually involves myelin-emergent nerve fibers which are intimately intermingled with non-myelin-emergent fibers. These are often enclosed at first by a single neurilemma. There is a progressive sorting out of such fibers so that the composite nerve containing both myelin-emergent and non-myelin-emergent fibers within a single neurilemma is transformed into a mixed nerve in which some of the constituent fibers are provided with individual sheaths.

Myelin segments, though relatively stable, may undergo various adjustments. Thus, end-to-end anastomosis of two segments may occur; a portion of a segment may be appropriated by the next one and a new node of Ranvier established; bare lengths of nerve fiber between two segments may become ensheathed by the process of intercalation of a whole segment.

Varieties of nerve regeneration and phenomena associated with nerve irritation and recovery in the salamander are quite like those already recorded in the frog tadpole.

Polariscopic observations of all stages of myelinogenesis indicate that the development of anisotropy closely parallels the development of the myelin sheath. The pre-myelin substance of prospective myelin

segments is isotropic; the youngest segments are weakly anisotropic; the older and thicker segments are strongly anisotropic.

By means of ciné-photomicrography, permanent records have been obtained which illustrate the essential changes exhibited by individual nerve fibers in salamanders during growth, regeneration, and myelination.

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